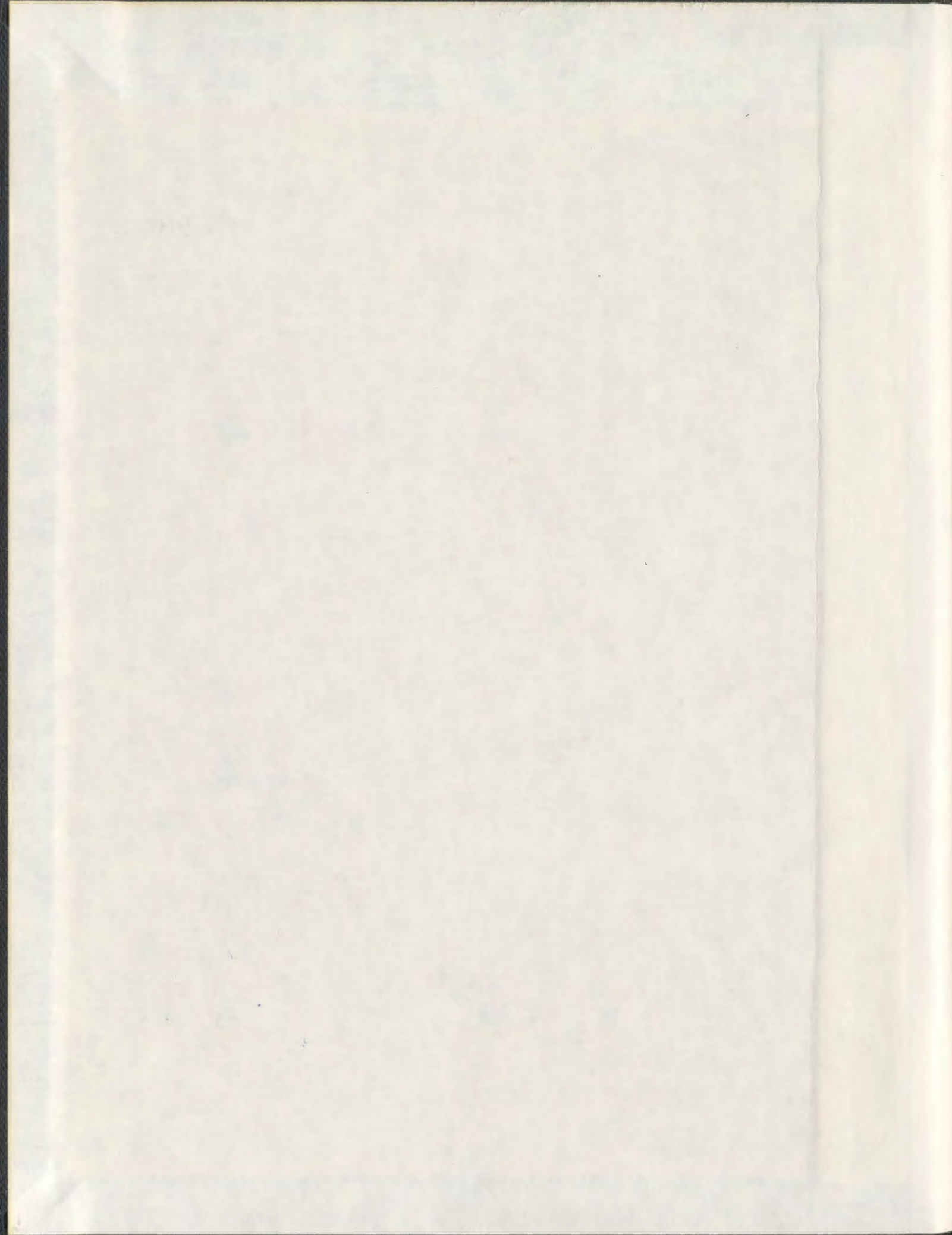


SIGNALLING PATHWAYS INVOLVED IN THE  
INHIBITION AND PROMOTION OF AXONAL  
REGENERATION

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Signalling Pathways Involved in the Inhibition and Promotion of Axonal Regeneration

By

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## ABSTRACT

Axonal regeneration following nerve injury requires the complex orchestration of various molecular events. Neurite outgrowth can be initiated by a variety of cues from the extracellular environment, including neurotrophins (e.g. nerve growth factor; NGF) and the extracellular matrix (ECM). Biological responses to neurotrophins are mediated by two distinct receptors: Trks, which initiate distinct signalling for the promotion of growth and survival, and p75NTR. Signalling pathways initiated by p75NTR exhibit considerable complexity and can elicit a variety of paradoxical physiological responses depending upon cellular context. Biological responses to ECM components, including laminin (LN), are mediated by a group of receptors known as integrins, which facilitate signal transmission to regulate cellular behaviour. Signalling pathways initiated by NGF and LN, have been previously reported to synergize resulting in optimized axonal regeneration of sensory neurons. The present series of studies sought to explore the molecular mechanisms underlying the enhanced growth initiated by stimulation with neurotrophins and LN, with a particular focus on the roles of the NGF receptors; specifically, TrkA phosphorylation-induced signalling cascades, and events associated with p75NTR ligand-dependent and independent signalling.

My initial studies utilized a series of PC12 cell derivatives expressing TrkA phosphorylation mutants, to investigate the potential role of TrkA in the regulation of p75NTR expression. I determined that TrkA played a role in the regulation of constitutive p75NTR expression, and further, controlled the upregulation of p75NTR in response to neurotrophin stimulation. In a subsequent study, I demonstrated that this occurred via a Phospholipase C  $\gamma$ -Protein Kinase C  $\delta$ -dependent mechanism, and confirmed the existence of this regulatory pathway in cerebellar granule neurons (CGN). I further investigated the contribution of the ECM to regenerative growth, in both its capacity to signal synergistically with the TrkA receptor for the



enhancement of early signalling intermediates, and its ability to elicit growth in a neurotrophin-independent scenario. Strikingly, integrin activation in the absence of neurotrophins was responsible for the promotion of neurite outgrowth via a rapid and potent Egr-1-dependent increase in the expression of the phosphatase PTEN, which relocalized to the nucleus where it dephosphorylated transcription factor Sp1, thereby decreasing its ability to bind to the p75NTR promoter, resulting in the subsequent downregulation of p75NTR and depression of Rho activity. This novel ECM-induced signalling paradigm was also determined to occur in CGNs, and following the development of a unique motility assay, I demonstrated that interference with this cascade impaired motility, suggesting that this signalling cascade may contribute to the developmental migration of CGNs.

p75NTR is a unique and flexible pleiotropic receptor which may promote or inhibit cell growth depending upon the presence or absence of neurotrophins. Taken together, the results of these studies detail the mechanisms involved in the upregulation of p75NTR expression in the presence of neurotrophins, but additionally present a novel signalling paradigm initiated by the ECM for the downregulation of the p75NTR in the absence of neurotrophin stimulation. Interestingly, both scenarios result in the promotion of neurite outgrowth and cellular motility as a result of the flexible signalling interactions of p75NTR, particularly those involving Rho, which can directly influence cytoskeletal dynamics.

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## LIST OF ABBREVIATIONS

Ab	Antibody
Akt	Protein kinase B
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ARA-C	Cytosine arabinoside
ARMS	Ankyrin rich membrane spanning protein
BDNF	Brain-derived neurotrophic factor
C3	Clostridium limosum exoenzyme C3
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene related peptide
chABC	Chondroitinase ABC
CNS	Central nervous system
Col	Collagen
CREB	cAMP response element binding protein
CSPG	Chondroitin sulphate proteoglycan
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DRG	Dorsal root ganglion
ECM	Extracellular matrix
ERK	Extracellular signal related kinase/ mitogen activated protein kinase
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FN	Fibronectin
FRS-2	Fibroblast growth factor receptor substrate 2
GAB1	Grb2 associated binding protein 1
GAG	Glycosaminoglycan
GAP	GTPase activating protein
GAP-43	Growth associated protein 43/ neuromodulin
GDI	Guanine dissociation inhibitor
GDNF	Glial cell line-derived neurotrophic factor
GEF	Guanine exchange factor
GPI	Glycosylphosphatidyl inositol
Grb2	Growth factor related binding protein 2
GSK3- $\beta$	Glycogen synthase kinase 3 $\beta$
HBSS	Hanks balanced salt solution
HRP	Horseradish peroxidase
IB4+	<i>griffonia simplicifolia</i> isolectin B4
ICC	Immunocytochemistry
Ig	Immunoglobulin
ILK	Integrin linked kinase
IP <sub>3</sub>	Inositol triphosphate
JNK	c-jun N-terminal kinase
K252a	receptor tyrosine kinase inhibitor
LN	Laminin
LY294002	Phosphatidylinositol 3 kinase inhibitor

mAb	Monoclonal antibody
MAG	Myelin associated glycoprotein
MAI	Myelin associated inhibitor
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase kinase
MG	Matrigel
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NADE	p75NTR associated death executor
NANS	Non-surface activated nanofibers
NF200	Neurofilament heavy chain
NGF	Nerve growth factor
NgR	Nogo receptor
NPC	Neural precursor cell
NRAGE	Neurotrophin receptor interacting MAGE homolog
NRIF	Neurotrophin receptor interacting factor
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5
OEC	Olfactory ensheathing cells
OMgp	Oligodendrocyte myelin glycoprotein
P75NTR	p75 neurotrophin receptor
PAK	p21 associated kinase
PBS	Phosphate buffered saline
PC12	Pheochromocytoma cell line
PDK	3'phosphoinositide dependent kinase
PI3K	Phosphatidylinositol 3 kinase
PIP	Phosphatidylinositol
PIP <sub>2</sub>	Phosphatidylinositol (4, 5)- biphosphate
PIP <sub>3</sub>	Phosphatidylinositol (3,4,5)-triphosphate
PH	Pleckstrin homology
PKA	Protein kinase A
PKB	Protein kinase B (Akt)
PKC $\delta$	Protein kinase C-delta
PL	Poly-D-Lysine
PLC $\gamma$	Phospholipase C-gamma
PNS	Peripheral nervous system
PP2	Src family kinase inhibitor
PSG	Penicillin/Streptomycin/Glutamine
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PTB	Phosphotyrosine binding domain
RGD	Arginine-glycine-aspartate
RIP	Regulated intramembrane proteolysis
Rock	Rho associated coiled coil kinase
Rsk	Ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SANS	Surface activated nanofibers
SC-1	Schwann cell factor 1
SDS	Sodium dodecyl sulfate
SFK	Src family kinase
SH2	Src homology 2



SH-SY5Y	Neuroblastoma cell line
SOS	Son of Sevenless
TBS	Tris buffered saline
TBS-T	Tris buffered saline plus tween-20
TNF	Tumour necrosis factor
TNF-R	Tumour necrosis factor receptor
TRAF-6	TNF receptor associated factor 6
Trk	Tropomyosin related kinase
TrkA	Tropomyosin related kinase A
TrkB	Tropomyosin related kinase B
TrkC	Tropomyosin related kinase C
UO126	MEK inhibitor
Y27632	Rock inhibitor

## **Chapter 1: Introduction**

Axonal regeneration following nerve injury requires the complex orchestration of a series of molecular events that allow the neuron to survive, re-grow and re-innervate the original target for functional recovery. The potential for axonal regeneration vastly differs between the peripheral nervous system (PNS) and the central nervous system (CNS), and regenerative ability is largely considered to be a consequence of the local microenvironment. The process of neurite outgrowth is heavily influenced by both growth-promoting and inhibitory factors in the extracellular environment, which bind receptors to exert their actions on the newly formed growth cone to promote extension or collapse respectively. As such, the vast differences in PNS and CNS microenvironments contribute to differential activation of signalling events that regulate regenerative outgrowth (reviewed in Busch and Silver, 2007; Huber et al., 2003; Lemons and Condie, 2008; Liu et al., 2006). Elucidating the signalling cascades initiated in the PNS which result in successful regenerative growth, both in terms of neurotrophin responsiveness and effects of permissive growth environments, is likely to provide insight into ways of enhancing CNS repair. The present series of studies sought to explore the molecular mechanisms underlying the enhanced neurite outgrowth initiated by stimulation with neurotrophins (including nerve growth factor) and extracellular matrix components (including laminin) in a sympathetic neuron model in order to optimize regenerative growth, and apply the consequent findings to the CNS.

## **1.1 Nervous System Injury**

The nervous system is a complex biological arrangement of interconnected neural networks which translate molecular cues from receptor ligations into electrical impulses to transmit signals reciprocally between the periphery and the brain for the regulation of complex human behaviours. While stability of this network is necessary to maintain higher order CNS function, this fixed wiring limits the ability of the adult mammalian CNS to recover from injury (Liu et al., 2006). The disruption of normal architecture by even small local injuries can result in devastating consequences and irreparable disability.

Individual neurons, which comprise the basic signalling unit of the nervous system, have a relatively simple morphology. Although a multitude of classifications exist, all neurons share several common features of basic architecture including a relatively small cell body and a single polarized axon that can extend considerable distances from the cell body and is responsible for the conduction of impulses. It is this unique geometry that renders neurons susceptible to axon damage (axotomy).

Nerve injury is heterogeneous in terms of both cause and outcome. Axotomy can be the result of physical injury (contusion, compression, penetration or transection), autoimmune disease, neurodegenerative disease, infection or diabetes, and it can result in a spectrum of pain, numbness, weakness or paralysis and/or a range of cognitive deficits with consequences dependent upon the severity, extent and location of the injury (reviewed in Okano et al., 2007; Thuret et al., 2006).

Acute CNS injury (eg. spinal cord injury) is essentially irreversible as functional recovery is severely limited, leaving the affected individual with diminished quality of life and immense costs associated with a lifetime of care (reviewed in Thuret et al., 2006).



The permanency of the damage is attributable to the restriction of axonal regeneration or plasticity imposed in part by the local CNS microenvironment. This is in contrast to the peripheral nervous system (PNS), where axonal regeneration following nerve injury occurs quite readily, yielding some functional recovery even in the adult, though it is often incomplete and rarely perfect (reviewed in He and Koprivica, 2004; Liu et al., 2006; Walmsley and Mir, 2007). Successful repair of the nervous system requires injured axons to survive, re-grow and reconnect with their original targets to mediate the recovery of function (reviewed in Bandtlow, 2003; Raivich and Makwana, 2007), all of which can occur to varying extents depending on the location and subset of neurons. Great strides have been made in our understanding of the barriers preventing CNS regeneration and a better, more complete understanding of components involved in promoting the successful PNS regenerative response may provide cues for strategies to initiate a regenerative response in the CNS.

### **1.1.1 Neuronal Response to Axotomy**

Axonal injury initiates a series of molecular events, cellular responses and ultrastructural changes, some of which are clearly involved in the mounting of a regenerative response in the PNS. Injury-induced stress signals arise from the loss of both neuronal activity (Al-Majed et al., 2000; Brushart et al., 2005) and trophic support from the innervation target (Raivich et al., 1991; Shadiack et al., 2001), and can be induced by the rapid influx of a variety of extracellular ions, including  $\text{Ca}^{++}$  and  $\text{Na}^{+}$ , through the transiently open membrane before it reseals (Berdan et al., 1993; Yoo et al.,

2003). These signals mediate early chromatolytic changes in axotomized neurons of the PNS: the cell body swells, the nucleus shifts to an eccentric position in the cell soma and the rough endoplasmic reticulum takes on a fragmented appearance (Lieberman, 1971). This is accompanied by metabolic changes including a dramatic increase in mRNA and protein synthesis, and alterations in gene expression. Axotomy induces the upregulation of adhesion molecules, transcription factors, structural components required for growth, and growth associated proteins (reviewed in Chen et al., 2007; Raivich and Makwana, 2007). Axotomy can also induce a rapidly elevated level of cyclic adenosine monophosphate (cAMP) in some populations of peripheral neurons (Neumann et al., 2002; Qiu et al., 2002b), which consequently enhances the intrinsic growth capacity of the neuron, possibly by upregulating polyamine synthesis, as polyamines have been demonstrated to stimulate neurite outgrowth both *in vitro* and *in vivo* (Oble et al., 2004; Schreiber et al., 2004; Sebille and Bondoux-Jahan, 1980). Furthermore, the injured axon, and surrounding glia, increase expression of growth factors, cytokines, neuropeptides and extracellular matrix molecules to create a favourable environment for regenerative growth in the PNS (reviewed in Raivich and Makwana, 2007). Axotomy induced molecular changes are largely reversible if regeneration is successful, as it often is in the PNS. However, if the regenerative response fails, as it often does in the CNS, it can lead to an extended period of apoptosis, demyelination, and synaptic stripping, wherein the innervation target atrophies and the synaptic input to the affected neuron withdraws, collectively resulting in cavitation and more distant effects (reviewed in Thuret et al., 2006).



These molecular events are accompanied by alterations to the cellular organization. Axotomy divides the axon into two segments. The distal segment is isolated from the cell body and responds to damage by retracting from its postsynaptic target and undergoing Wallerian degeneration (Becerra et al., 1995), a process in which recruitment of phagocytic macrophages, working in concert with Schwann cells, leads to the clearance of myelin fragments and axon-derived debris (Chen et al., 2007; Makwana and Raivich, 2005). While the distal portion of the axon itself degenerates, connective tissue often remains, creating a distal stump. The proximal segment remains attached to the cell body and if the cell survives the insult, the  $\text{Ca}^{++}$  influx initiates cytoskeletal remodelling for the formation of a growth cone at the severed edge, which appears fundamentally equivalent to those of developing fibres (Spira et al., 2001), though evidence is emerging to suggest that development and regenerative growth are distinct processes (reviewed in Zhou and Snider, 2006). Axonal sprouts from the proximal segment can enter the distal stump and re-extend toward the original target, guided by environmental factors that promote growth cone movement in the developing nervous system. This process is quite successful in all three branches of the PNS (motor, sensory and autonomic). Even in the CNS, proximal stumps form growth cones and sprout, but long distance regeneration is rare, rendering CNS injury irreversible.

### **1.1.2 Signalling at the Growth Cone**

Axons are required to extend long distances during both developmental and regenerative growth (Zhou and Snider, 2006). Axonal extension is a highly regulated



process in which outgrowth, guidance and branching are carefully orchestrated and a trajectory is determined by the deployment of spatially and temporally regulated extracellular factors which act on the growth cone at the leading tip of the growing axon (Dickson, 2002; Huber et al., 2003; Song and Poo, 2001; Tessier-Lavigne and Goodman, 1996). The growth cone is a dynamic and motile structure. It serves as the major site of reception and interpretation of extracellular cues that direct cytoskeletal changes and rapidly responds to these cues to acutely alter axon behaviour. As such, the growth cone is responsible for the interpretation of a fine balance of attractive and repulsive cues to achieve an intricate pattern of innervations (Huber et al., 2003).

The growth cone is comprised of two elements: filopodia are finger-like projections of bundled F-actin, which constantly probe the extracellular terrain in search of growth and guidance cues, and lamellipodia are broad webs of cross-linked actin filaments, which also play a role in detecting the extracellular environment (Figure 1.1 A). Growth cone advance initiates axonal elongation via actin polymerization at the leading edge, the retrograde flow of F-actin, and the depolymerization of actin at the opposite end for a treadmilling effect. Growth promoting molecules enhance actin polymerization and prevent the retrograde flow of actin resulting in the forward advance of the growth cone. Subsequent microtubule polymerization and bundling within the core complete the formation of a newly formed axon segment. Repulsive cues are just as important to axon guidance and can result in collapse of the growth cone and axon retraction by promoting actin depolymerization and enhancing the retrograde actin flow (Huber et al., 2003; Mallavarapu and Mitchison, 1999; Mitchison and Kirschner, 1988; Suter and Forscher, 2000).



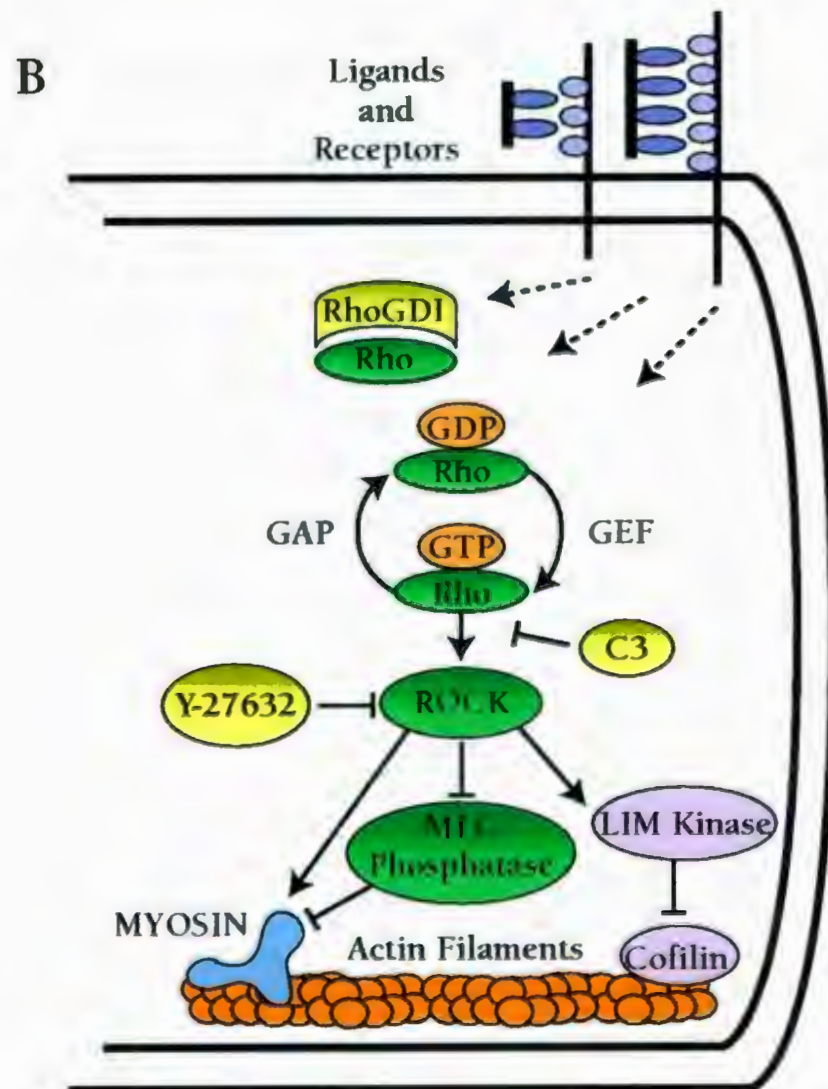
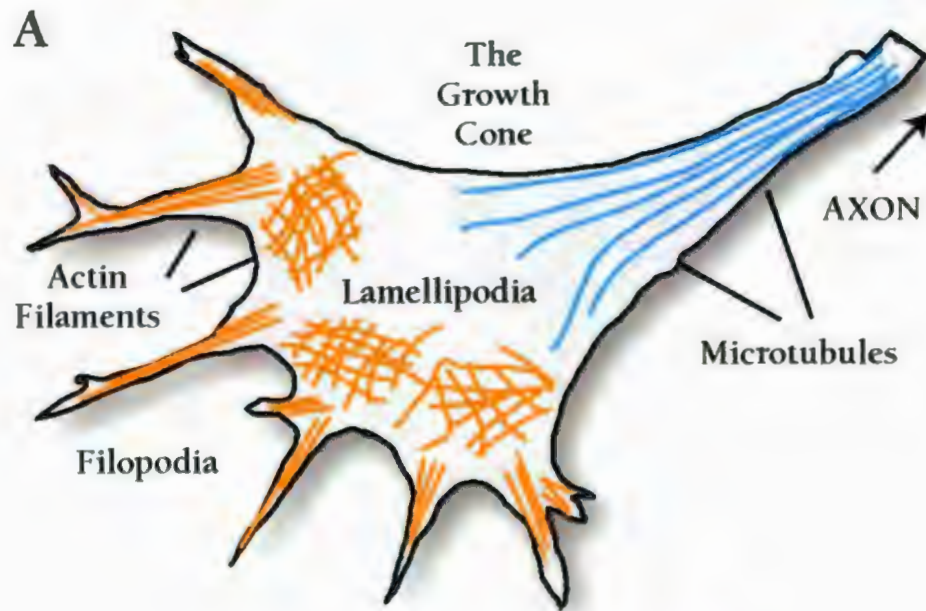


Figure 1.1



The initiation of axon growth requires specific stimulation of the growth cone by factors in the extracellular environment which are transduced to organize local axon assembly, a process which involves a coordinated regulation of gene expression in the nucleus, protein expression in the soma and along the length of the axon, the anterograde transport of raw materials and the assembly of the cytoskeletal elements into new axons (Zhou and Snider, 2006). The response of the growth cone to extracellular growth and guidance cues is dependent upon the intracellular state of the growth cone, and the presence of an adhesive and permissive substrate.

A variety of extracellular molecules, including short, intermediate and long range target-derived factors, are recognized to influence axon extension (Huber et al., 2003). As well, growth-inducing and trophic molecules are released following injury to act on the proximal tip to induce regenerative growth (reviewed in Chen et al., 2007; Raivich and Makwana, 2007). The final changes that occur in response to these extracellular signals, whether the signals trigger or inhibit growth, are changes to the cytoskeleton. These diverse signals translate into cytoskeletal changes leading to axon extension by convergence of intracellular signalling pathways on a common set of effectors that mediate cytoskeletal dynamics. Cytoskeletal assembly is a highly conserved process that is regulated by a common set of molecules across cell types and species: the family of proteins known as Rho GTPases (Dickson, 2001; Hall, 1998; Luo, 2000; Luo, 2002).

### 1.1.2.1 Rho GTPases

Rho GTPases comprise a family of proteins that serve as molecular switches to regulate cytoskeletal structure and dynamics. Any signalling cascade that has an effect on growth cone dynamics must ultimately effect changes to the cytoskeleton (Filbin, 2003). Rho GTPases play an essential role in translating extracellular signals into changes in cytoskeletal proteins, and can transduce signalling pathways stimulated by receptor-ligand interaction to cytoskeletal changes in the growth cone by serving as convergence points which exert control over the dynamics of actin polymerization and depolymerisation, thus regulating actin filament assembly or disassembly. These proteins thereby possess the ability to modulate varied cellular responses including morphological changes, neurite outgrowth, proliferation, adhesion, phagocytosis and migration (Dickson, 2001; Hall, 1998; Luo, 2000; Luo, 2002).

Rho GTPases cycle between GTP-bound active and GDP-bound inactive forms. This cycling is regulated by the opposing activities of guanine nucleotide exchange factors (GEF) and GTPase activating proteins (GAP). GEFs facilitate the exchange of GDP for GTP thereby activating Rho GTPases and allowing them to recruit effector proteins. GAPs activate the endogenous GTPase activity resulting in hydrolysis of GTP to GDP, thus disrupting the interaction with effectors and resulting in inactivation (Figure 1.1 B). A third regulatory element, guanine dissociation inhibitors (GDI) sequester inactive Rho and inhibit formation of active Rho-GTP. When GTP-bound, Rho GTPases can interact with intracellular effectors to reorganize the cytoskeleton both by regulating actin filament assembly/disassembly through polymerization control and by directing



actin/myosin contractility to control retrograde transport of F-actin in the growth cone (reviewed in Dickson, 2001; Hall, 1998; Huber et al., 2003; Luo, 2000; Luo, 2002).

Receptors modulate the activity of GTPases by coupling directly or indirectly to GEFs and GAPs upon ligation.

The best known family members, Cdc42, Rac and Rho all play roles in regulation of lamellipodial and filopodial dynamics. Cdc42 and Rac mediate growth cone attraction and growth by promoting extension of the filopodia and lamellipodia respectively.

Conversely, Rho activity causes growth cone repulsion and collapse by promoting actin depolymerisation in the growth cone. GTPase actions are exerted through interactions with downstream effectors. Cdc42 effectors include N-WASP, a key regulator of the Arp2/3 complex which controls actin filament nucleation and branching (Higgs and Pollard, 2001) and thereby promotes actin polymerization. Actin polymerization is also regulated by profilin, which controls the addition of actin monomers to the plus end of actin filaments (Suetsugu et al., 1998), working with proteins of the VASP/Ena family that prevent actin capping. Cdc42 and Rac effector, p21-activated kinase (PAK) prevents retrograde flow of actin by dephosphorylating myosin (Kamm and Stull, 1985). These signalling interactions collectively contribute to promotion of growth.

Conversely, Rho effector protein, Rho associated kinase (ROCK) activates LIMK which inhibits the activity of cofilin (actin depolymerizing factor) thereby inhibiting actin filament disassembly and preventing the necessary actin treadmilling necessary for growth (Arber et al., 1998). ROCK also promotes actin-myosin contractility and retrograde flow of F-actin through the phosphorylation of myosin, also resulting in the inhibition of neurite outgrowth (Amano et al., 1996) (Figure 1.1 B). Not surprisingly,



receptors for multiple classes of growth cone chemorepellents (Eph/ephrins, plexin/semaphorins, Robo/slit) cause growth cone collapse by activating Rho (reviewed in Huber et al., 2003) and the inhibition of Rho or ROCK results in improved regenerative growth (Borisoff et al., 2003; Ellezam et al., 2002; Fournier et al., 2003). Thus since Rho GTPases are central regulators of actin cytoskeletal modulation and retrograde F-actin flow, processes essential for extension of regenerating axons, they are central players in the success and failure of the growth process.

## **1.2 Inhibition of Adult CNS Axonal Regeneration**

In the adult nervous system, there exists a striking dichotomy in regenerative ability between the CNS and the PNS. Neurons subjected to axotomy in the PNS can mount a vigorous regenerative reaction, while those axons injured in the CNS fail to mount a regenerative response regardless of where the cell body resides (eg. the central branch of the dorsal root ganglion (DRG) axon fails to regenerate despite the fact that the cell body is located in the PNS). The failure of nerves to regenerate in the CNS is traditionally ascribed to two limiting factors: an inhospitable environment and the neuron's response to that environment, a phenomenon referred to as intrinsic growth capacity (reviewed in Domeniconi and Filbin, 2005; Liu et al., 2006). Not only is the CNS microenvironment relatively devoid of growth promoting neurotrophic factors, but it is also abundantly rich in inhibitory molecules associated with myelin debris which is cleared far more slowly in the CNS than in the PNS (David et al., 1990). Additionally, glial cells unique to the CNS undergo changes that are counterproductive to regenerative

growth in the CNS including the formation of an impenetrable physical barrier known as the glial scar and the secretion of growth inhibitory chondroitin sulphate proteoglycans (reviewed in David and Lacroix, 2003; Liu et al., 2006). Finally, the intrinsic state of the adult CNS neurons is less conducive to a regenerative growth response, relative to their neonatal or PNS counterparts (Neumann et al., 2005; Qiu et al., 2002a; Qiu et al., 2002b). These intrinsic and extrinsic factors are not mutually exclusive entities, as intrinsic capacity can be modulated by extracellular cues (David and Aguayo, 1981; Goldberg et al., 2002; Richardson et al., 1980) and the response to extracellular cues is dependent upon the intrinsic state of the neuron (Bandtlow, 2003). Because the intrinsic capacity and extrinsic cues for growth are invariably linked, I will briefly discuss the roles of each.

### **1.2.1 Intrinsic Factors Inhibiting CNS Regeneration**

Normal development of the CNS is reliant upon accurate intrinsic cellular programs in addition to extrinsic informative cues provided by the extracellular environment. Regenerative growth occurs under the same restraints and may be dictated by similar parameters. The regenerative capacity of the adult mammalian CNS is extremely limited (reviewed in Okano et al., 2007), and this is not solely a consequence of the local microenvironment, but also seems to be a property acquired with maturation. The CNS of young animals can undergo successful regeneration (Bregman et al., 1989; Fry et al., 2003; Hasan et al., 1993; Saunders et al., 1998; Shimizu et al., 1990), which suggests developmental changes are central to the loss of this response. The regenerative ability of young neurons is lost during late postnatal development, a time period

corresponding to the onset of myelination (Bandtlow, 2003; Keirstead et al., 1992); however environmental changes are not solely responsible for the loss (Chen et al., 1995a; Dusart et al., 1997; Goldberg et al., 2002; Li et al., 1995). When cultured under identical conditions, adult CNS neurons extend more slowly than their neonate or PNS counterparts (Blackmore and Letourneau, 2006). Furthermore, young neurons respond differently to inhibitors in myelin and are capable of extending through the adult CNS environment (Blackmore and Letourneau, 2006). The response to extracellular cues is clearly regulated by the intracellular state of the neuron and loss of the ability to regenerate may in fact be due to target-derived stop signals delivered after the neurons form synaptic connections; changes which fail to reverse after axotomy (Zhou and Snider, 2006). Regardless of origin, the loss of intrinsic growth capacity is likely mediated by intracellular levels of cyclic adenosine monophosphate (cAMP) (Cai et al., 2001).

cAMP levels are endogenously high in young neurons and levels fall sharply with age coinciding with the loss of regenerative ability (Domeniconi and Filbin, 2005; Filbin, 2006). cAMP levels are dramatically increased in peripheral neurons after injury, but not in central neurons (Neumann et al., 2002; Qiu et al., 2002a; Qiu et al., 2002b), and alterations in cAMP levels can dictate the response of a growing axon to guidance cues, neurotrophic factors and myelin associated inhibitory proteins; high cAMP levels favour attraction, and low cAMP levels favour repulsion (Song et al., 1998; Song et al., 1997). The intrinsic state of the neuron can thus be manipulated by injection of cAMP, cAMP analogues (db-cAMP) or by a conditioning lesion, a phenomenon wherein a lesion to the peripheral branch of the DRG results in elevated cAMP triggering a regenerative response to a later injury of the central branch which normally would not be capable of



regenerative growth (Cai et al., 2001; Kao et al., 2002; Lu et al., 2004; Neumann et al., 2002; Qiu et al., 2002a; Qiu et al., 2002b). Artificial elevation of intracellular cAMP levels can also induce adult CNS neurons to regenerate despite the presence of myelin associated inhibitory molecules (Neumann et al., 2002; Qiu et al., 2002a; Qiu et al., 2002b), and blocking cAMP can render young neurons susceptible to inhibition by myelin (Domeniconi and Filbin, 2005).

cAMP signalling proceeds via protein kinase A (PKA) which may regulate cytoskeletal organization by inactivating Rho for immediate growth cone collapse (Qiu et al., 2002a; Snider et al., 2002). It also induces Arginase I transcription and subsequent polyamine synthesis, both reported to promote regenerative growth in the PNS (Cai et al., 2002). Intrinsic regenerative capacity can therefore be restored by blocking Rho activity, increasing cAMP levels or delivery of neurotrophic factors via infusion, hybridoma graft or viral transduction which are also associated with elevated cAMP levels (reviewed in (Fawcett, 2002).

Maturation events clearly contribute to the diminished growth capacity of adult mammalian CNS neurons. Notwithstanding, these neurons do retain adequate potential to extend long axons if presented with a favourable environment, rich in neurotrophic factors and permissive extracellular matrix molecules (Benfey and Aguayo, 1982; David and Aguayo, 1981), suggesting that the CNS microenvironment plays a key role in the inhibition of regenerative growth.

### **1.2.2 Extrinsic Factors Inhibiting CNS Regenerative Growth**

Although multiple factors are likely to underlie the failure of adult CNS neurons to regenerate, there is little doubt that the developmentally regulated changes to the CNS microenvironment play a key role. The fact that isolated adult CNS neurons can be encouraged to extend long processes *in vitro* suggests that the failure to regenerate is not solely attributable to the lack of intrinsic growth ability, but is also a function of a poor extracellular microenvironment, devoid of growth promoting factors, but instead containing an adverse glial reaction and multiple inhibitory molecules (Benfey and Aguayo, 1982; Crutcher, 1989; David and Aguayo, 1981; David and Lacroix, 2003; Richardson et al., 1980; Schnell and Schwab, 1990).

#### **1.2.2.1 The Glial Scar**

Injury to the CNS results in the formation of a glial scar, a dynamic and dense impenetrable barrier of scar tissue which surrounds the site of the lesion, usually oriented perpendicular to the neuraxis, and serves to reseal the blood brain barrier and compact inflammatory cells (Berry et al., 1983). The glial scar is largely comprised of astrocytes that have undergone reactive gliosis, a reaction in which cells enlarge and extend hypertrophic interdigitating processes to enmesh the lesion site. Injury-induced disruption of the blood brain barrier results in the invasion of non-CNS factors including those involved in the inflammatory response (Fitch et al., 1999; Preston et al., 2001), but also fibroblasts which invade the lesion core and induce the astrocytic expression of repulsive guidance cues including semaphorins, eph/ephrins, and slit, which act to repel

axons (Bundesen et al., 2003; De Winter et al., 2002; Goldshmit et al., 2004; Hagino et al., 2003; Pasterkamp et al., 1999). Thus the glial scar is not simply a physical barrier, but also contains multiple secreted and transmembrane molecular inhibitors of axonal growth, which induce the formation of dystrophic growth cones.

Notably, reactive astrocytes express several types of chondroitin sulphate proteoglycans (CSPG) including brevican, phosphacan, aggrecan, neurocan and NG2, and this production is exacerbated by the inflammatory reaction (Bandtlow and Zimmermann, 2000; Dou and Levine, 1994; Fawcett and Asher, 1999; Jones et al., 2002). CSPGs have been demonstrated to mediate inhibition of axon growth via the glycosaminoglycan (GAG) chains (Chen et al., 2002; Dou and Levine, 1994). CSPGs are clearly inhibitory but largely unknown in terms of receptor and signalling, although it is clear that activation of the Rho-ROCK signalling cascade is a necessary step in mediating CSPG-associated inhibition *in vitro* (Borisoff et al., 2003; Dergham et al., 2002; Monnier et al., 2003), and it has been suggested that CSPGs interfere with integrin signalling as integrin overexpression can promote axon growth despite the presence of CSPGs (Condic, 2001).

The formation of the glial scar is essential to seclusion of the damaged site and protection of surrounding area from secondary injury, as genetic ablation of reactive astrocytes prevents the blood brain barrier from resealing leading to increased lesion size, enhanced cell death, and exacerbated immune response (Faulkner et al., 2004).

Fortunately, the glial scar is not an insurmountable obstacle. While the development of the scar begins within hours of injury, it can take as long as two weeks to fully mature (Berry et al., 1983), presenting a window of opportunity for axonal regeneration.

Furthermore, the inhibitory effects of the scar can be overcome by enzymatic removal of



CSPGs using chondroitinase ABC (chABC) (Bradbury et al., 2002; Moon et al., 2001), and inhibition of Rho activity downstream of CSPG exposure resulted in a significant but limited number of axons growing across the scar (Dergham et al., 2002; Winton et al., 2002).

#### **1.2.2.2 CNS Myelin**

The non-permissive nature of the CNS microenvironment was first appreciated by Cajal in 1927, and the axon growth inhibition associated with myelin components is a significant contributor to this hostile environment. Indeed the onset of myelination correlates temporally with the end of the regeneration permissive period during development (Bregman et al., 1993; Hasan et al., 1993; Kalil and Reh, 1982; Saunders et al., 1998; Tolbert and Der, 1987; Treherne et al., 1992).

CNS myelin is produced by mature differentiated oligodendrocytes, and the process of axon myelination occurs late in development, generally after the growth cone has reached its target. Intact myelin insulates the axon and allows for saltatory conduction, but myelin can be disrupted by injury resulting in myelin debris strewn about the lesion site. This debris is cleared through the process of Wallerian degeneration. However, while Wallerian degeneration occurs rapidly in the PNS, it is a relatively slow process in the CNS, where it can take months to years to adequately remove myelin fragments and is questionably ever complete (reviewed in David and Lacroix, 2003; Filbin, 2006).

The concept of myelin inhibition was explored by Berry (1982) who demonstrated that axons of the CNS could regenerate successfully following chemical axotomy that doesn't damage myelin, but not following a mechanical injury. Similarly, atraumatic transplantation of adult DRG into white matter can result in successful regenerative growth, providing that the myelin is not disrupted (Davies et al., 1997; Davies et al., 1999). Schwab and colleagues identified damaged CNS myelin as a substrate inhibitory to the regeneration of injured CNS axons (Caroni and Schwab, 1988a; Caroni and Schwab, 1988b; Savio and Schwab, 1989; Schwab and Thoenen, 1985). Indeed, CNS myelin homogenates are capable of inducing growth cone collapse *in vitro* (Bandtlow et al., 1990; Savio and Schwab, 1989). Conversely, PNS myelin produced by Schwann cells does not possess potent neurite outgrowth inhibitory activity (Carbonetto et al., 1987; Crutcher, 1989; Khan et al., 1990; Sagot et al., 1991; Savio and Schwab, 1989).

#### **1.2.2.2.1 Myelin Associated Inhibitory Proteins**

The search for molecular components present in CNS myelin that impair regenerative growth led to the identification of three molecules that act as potent neurite growth inhibitors *in vitro*: Nogo, Myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (Caroni and Schwab, 1988a; Caroni and Schwab, 1988b; Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000; Spillmann et al., 1998).

NogoA is the largest of the 3 Nogo isoforms, and is highly expressed by oligodendrocytes of the CNS, but not by Schwann cells of the PNS, consistent with its

role as a myelin associated inhibitor (Liu et al., 2006). Structurally, NogoA has two transmembrane domains flanking a 66 amino acid extracellular loop (GrandPre et al., 2000; Huber and Schwab, 2000). Functionally it has two known inhibitory domains capable of inducing growth cone collapse: the 66 amino acid extracellular loop (Nogo66) and the N-terminal region (amino-Nogo) (Chen et al., 2000; Fournier et al., 2001; Prinjha et al., 2000). NogoA potently inhibits axonal regeneration in the damaged CNS, and may function in the normal CNS to prevent abnormal sprouting.

MAG is a member of the immunoglobulin superfamily. It is expressed by both CNS and PNS myelinating glial cells, but is ten-fold more abundant in the CNS (Trapp, 1988; Trapp, 1990), and is upregulated by CNS injury (Mingorance et al., 2005). It possesses both adhesive and inhibitory properties dependent upon the age of the neuron; MAG promotes axonal growth from young neurons but potently inhibits regenerative growth from older cells (Cai et al., 2001; DeBellard et al., 1996). Furthermore, it plays a key role in the maintenance of myelin architecture and limits plasticity (Schachner and Bartsch, 2000).

OMgp is another myelin associated protein that is localized to the cell surface. It also possesses potent growth cone collapsing activity and thus prevents neurite outgrowth (Kottis et al., 2002; Wang et al., 2002b).

The contribution of each myelin component to the inhibition of axonal extension is dependent upon several factors, including the relative abundance of each component, and therefore the likelihood of growth cone contact, and the presence of all components of the receptor complex.



#### **1.2.2.2.2 Myelin Associated Inhibitor Protein Receptors and Signal Transduction**

While the receptor for amino-Nogo has yet to be identified, the receptor for Nogo-66 was identified by Strittmater and colleagues in 2001 to be a protein now known as the Nogo receptor (NgR) (Fournier et al., 2001). It was further demonstrated that a direct interaction between Nogo-66 and NgR was necessary to induce growth cone collapse (Fournier et al., 2001). The other myelin proteins, MAG and OMgp, lack sequence similarity or domain homology to Nogo-66, but were nonetheless shown to bind NgR to exert their effects as well, though this activity was linked to separate binding sites of NgR (Domeniconi and Filbin, 2005; Kottis et al., 2002; Liu et al., 2002; Wang et al., 2002b). NgR crystallization elucidated the ability of this receptor to bind diverse ligands by illustrating the presence of numerous different binding platforms (Barton et al., 2003), and this redundancy explains the poor efficacy of the therapeutic blockade of only a single component of myelin. The forced expression of NgR in myelin unresponsive cells can render them responsive to myelin inhibition (Fournier et al., 2001). In contrast, dominant negative forms of NgR, or siRNA mediated suppression of NgR can dramatically decrease the inhibitory effects of myelin (Ahmed et al., 2005; Domeniconi et al., 2002; Wang et al., 2002a; Wang et al., 2002b), and DRG lacking NgR do not show growth cone collapse in the presence of myelin associated inhibitors (Kim et al., 2003).

NgR is a glycosyl phosphatidylinositol (GPI)-linked protein and therefore possesses no transmembrane or cytoplasmic intracellular signalling domains. As a result, NgR has distinct inability to transduce signals from myelin across the membrane, and thus a second molecule is required for the transduction of inhibitory signals. The transducing element of this receptor complex was identified to be p75NTR, the low

affinity neurotrophin receptor (Wang et al., 2002a). As such, the partial genetic deletion of p75NTR exon III or the overexpression of a truncated version in neurons removes the ability of myelin to inhibit growth from these cells (Wang et al., 2002a; Yamashita et al., 2002). However, axonal regeneration of all neurons is inhibited by myelin, but not all neurons express p75NTR. In many neurons, p75NTR expression can be induced by injury (Martinez-Murillo et al., 1998). Alternatively, TAJ/TROY, another tumour necrosis factor (TNF) superfamily member expressed in many adult neurons, can substitute for p75NTR and serve as an alternate co-receptor (Park et al., 2005; Shao et al., 2005). A third component of this receptor complex is LINGO-1, which is believed to promote the association of ligand-bound NgR and transducing partner p75NTR (Mi et al., 2004).

Myelin-induced growth cone collapse via NgR-p75NTR receptor complex induces cytoskeletal changes, and as such, likely involves the key regulators of actin dynamics, the Rho GTPases. In fact, Rho was implicated in mediating the effects of myelin associated inhibitory proteins before NgR was even discovered (Lehmann et al., 1999). Exposure to myelin results in increased activity of Rho in neurons (Fournier et al., 2003; Schweigreiter et al., 2004; Winton et al., 2002) and the inactivation of Rho or downstream effector ROCK using dominant negative forms or a cell permeable inhibitor blocks the effects of myelin and allow neurites to grow over inhibitory substrates (Ellezam et al., 2002). Stimulation of Rho activity is therefore critical for myelin associated signal transduction downstream of NgR, as is activation of Rho effector ROCK and consequent actinomyosin contractility which initiates growth cone collapse and neurite retraction. Myelin associated inhibitors also inactivate Rac, although it

remains unclear whether this is necessary or sufficient to prevent myelin associated inhibition (Niederost et al., 2002). Nonetheless, NgR-p75NTR complex signalling through Rho-ROCK has implications for axonal regeneration in the adult mammalian CNS. The inactivation of Rho or ROCK activity promotes axonal growth despite the presence of myelin associated inhibitory proteins and in some cases has led to functional recovery (Dergham et al., 2002; Fournier et al., 2003; Lehmann et al., 1999).

### **1.2.3 Therapeutic Intervention**

Tremendous increases in our understanding of the barriers preventing regeneration in the CNS and why this ability differs in adult PNS and embryonic/neonatal neurons of both systems, have in turn lead to the development of multiple strategies designed to overcome barriers, including a poor extracellular environment, and enhance axonal regeneration for therapeutic recovery of function. Multiple strategies have proven effective in protecting cells from secondary cell death and promoting varying amounts of regenerative growth (reviewed in David and Lacroix, 2003; Fawcett, 2002; Filbin, 2006; Thuret et al., 2006). The majority of the proposed strategies attempt to subvert the inhibition imposed by the CNS microenvironment by blocking inhibitors or their receptors, or by altering the neuronal response to the inhibitors via alteration of the neuron's interpretation of inhibitory cues. In this regard, the intrinsic growth potential of CNS neurons can be manipulated by exogenous elevation of cAMP levels, or by inactivation of Rho, both of which can be accomplished by the infusion of neurotrophic factors (reviewed in David and Lacroix, 2003; Fawcett, 2002). Neurotrophic factors are



however, not solely effective as they often result in unacceptable side effects, and chronic injury is often associated with a gradual loss of growth factor receptors (Kwon et al., 2004).

Strategies aimed specifically at overcoming the inhibition of the glial scar include enzymatic digestion of inhibitory CSPGs using chondroitinase, which has been shown to permit the extension of axons into the glial scar (Bradbury et al., 2002; Moon et al., 2001). Furthermore, the transplantation of olfactory ensheathing cells (OECs) derived from the regeneration permissive olfactory system, were able to migrate through the glial scar carrying axons with them to mediate regenerative growth (Li et al., 1998b; Ramon-Cueto et al., 2000).

A comprehensive effort to overcome the inhibition associated with a myelin rich environment of the CNS has led to the development of multiple blocking antibodies and peptides directed at the myelin associated inhibitory proteins themselves, and their receptor complex, and have resulted in varying degrees of success, thwarted in part by the redundancy of the ligands for the NgR-p75NTR receptor complex (reviewed in David and Lacroix, 2003; He and Koprivica, 2004).

Successful regeneration has also been achieved by the provision of growth promoting extracellular factors, generally in the form of tissue grafts. David and Aguayo (1982) demonstrated that the transplantation of a segment of peripheral nerve was sufficient to encourage the successful regenerative growth of retinal neurons to yield functional connections. Peripheral nerve grafts have since been shown to support the ingrowth of various axonal types in the CNS (reviewed in He and Koprivica, 2004; Thuret et al., 2006). Grafts of Schwann cells, a prominent PNS glial type, are reported to

promote the extension and remyelination of spinal axons (Bamber et al., 2001; Takami et al., 2002; Xu et al., 1995a; Xu et al., 1995b) which subsequently become electrophysiologically active (Pinzon et al., 2001). As previously mentioned, grafting of OECs can promote CNS regeneration, though not under all circumstances, and fetal spinal cord transplantation has yielded some modest success (Bregman et al., 1993; Jakeman and Reier, 1991; Kunkel-Bagden and Bregman, 1990; Reier et al., 1992). Whether these grafts are acting as relays or conduits or simply providing permissive cues that signal to promote outgrowth, the improved extracellular environment promotes regenerative growth and functional recovery, but can frequently suffer from a graft-host barrier in which the re-extended axons fail to exit the graft and re-enter the host environment, a key step to reformation of functional synapses for the recovery of function. However, this graft-host interface can be overcome by the provision of growth factors (Menei et al., 1998; Xu et al., 1995a), which are also an abundant component of the PNS and recognized to contribute to the permissive nature of the PNS environment.

Relative to the PNS, where more than 90% of axons can be induced to regenerate under optimal conditions, experimental intervention in the CNS promotes the regenerative growth of only a small percentage of the original fibre tract (Bradbury and McMahon, 2006), and the distance the CNS axons are able to extend is severely limited. For example, in one study, Nogo neutralization was reported to induce only 5% of the corticospinal tract axons to regenerate after injury, and in general less than 10% of central neurons will regenerate at least two spinal segments (4-6 mm) enough to be of functional use (Bradbury et al., 2002; Bregman et al., 1995; GrandPre et al., 2002). Surprisingly, this minor amount of re-growth is sufficient to yield a disproportionate return of function,



likely owing in part to the same mechanisms that allow for more than 80% axon loss before deficits become apparent (Coumans et al., 2001; Fawcett, 2002).

Each of the strategies discussed here have produced significant regeneration with variable degrees of success when applied to rodent CNS injury models. It is apparent that multiple factors can act synergistically to promote regenerative growth and that combination strategies will be necessary for optimal recovery of function. The majority of studies to date promote regenerative growth by altering the extracellular environment. While it remains unclear if the mechanisms regulating PNS regenerative growth are relevant to the CNS situation and can be harnessed to promote CNS growth after injury, it is necessary and important to determine how the permissive environment of PNS impacts intracellular signalling and consequent regenerative behaviour, which will be the focus of this project.

### **1.3 The Promotion of PNS Axonal Regeneration**

In contrast to the CNS situation, PNS axotomy is often followed by a spontaneous and successful regenerative response. PNS injury results in the elevation of intracellular cAMP levels, thereby activating the intrinsic growth program (Qiu et al., 2002b). This is well demonstrated by the aforementioned conditioning lesion of the peripheral branch of the DRG, which can subsequently induce the regenerative growth of the central process into the CNS (Neumann and Woolf, 1999; Richardson and Issa, 1984). These effects can be mimicked by the infusion of cAMP analogues (db-cAMP) (Neumann et al., 2002; Qiu et al., 2002a; Qiu et al., 2002b). However, activation of the intrinsic growth program will



increase regenerative ability but functionally successful regeneration remains dependent on the availability of a positive and substantiating substrate, specific stimulatory signals and/or the existence of axon guidance cues to direct axonal growth towards an appropriate target. As such, successful regenerative growth of the PNS is largely attributable to the local extracellular environment and the presence of permissive molecules therein. The PNS microenvironment is rich in soluble neurotrophic factors and the extracellular matrix (ECM) is comprised largely of laminins (LN) (reviewed in Chen et al., 2007). Furthermore, the PNS is devoid of astrocytes and expresses vastly fewer proportion of inhibitory proteins than the CNS, including tenfold less MAG (Trapp, 1990), and any injury induced debris is rapidly cleared from the PNS by Wallerian degeneration.

The major glial component of the PNS is Schwann cells, which both ensheath and myelinate peripheral axons and form a continuous basal lamina. Schwann cells respond to injury by de-differentiating and undergoing a proliferative reaction, subsequently becoming migratory and forming into parallel alignment to create permissive tubes known as Bands of Bungner (Fawcett and Keynes, 1990). They also secrete a variety of neurotrophic factors, as well as LN, an ECM component that is permissive to neurite outgrowth (Chiu et al., 1991). The deposition of permissive ECM components such as LN, and the availability of neurotrophic factors contribute to the creation of a favourable environment for the promotion of axonal regeneration. Determination of the cellular effects of these growth promoting molecules may reveal why these PNS neurons are able to regenerate spontaneously and successfully.

### 1.3.1 The Role of Neurotrophins in Promoting PNS Regenerative Growth

Neurotrophins are a small group of dimeric growth factors implicated in the development and maintenance of different populations of neurons. NGF is the prototypic member of a highly conserved family derived from a common ancestral gene (reviewed in Huang and Reichardt, 2001), which also includes BDNF, NT-3 and NT-4/5. NGF was originally characterized for its ability to stimulate growth, differentiation, survival and maintenance of peripheral sensory and sympathetic neurons during development and after injury (reviewed in Levi-Montalcini, 1987), but has since been appreciated to regulate many aspects of neuronal development and function including myelination, apoptosis, proliferation and plasticity (Arevalo and Wu, 2006; Bibel and Barde, 2000; Hennigan et al., 2007; Kaplan and Miller, 2000; Lewin and Barde, 1996; Lu et al., 2005; Sofroniew et al., 2001).

During development and beyond, neurotrophins are produced and constitutively secreted by targets of sensory and sympathetic innervations, including skin, muscle, and endocrine tissue. Neurotrophins can also be secreted by non-neuronal cells, and by sympathetic and sensory neurons themselves to exert paracrine or autocrine actions (Levi-Montalcini et al., 1995; Levi-Montalcini et al., 1996; Reichardt, 2006). Transiently required growth factors may also derive from sources other than the final target, including intermediate targets that axons encounter and extend beyond while *en route* to their final destination to provide trophic support to neurons that have not yet contacted their final targets (Farinas et al., 1998; Farinas et al., 1996; Huang et al., 1999; Ringstedt et al., 1999). Neurotrophin expression can be modulated by neuronal electrical activity (Castren et al., 1992; Dragunow et al., 1993; Patterson et al., 1992; Rocamora et al., 1996), or in



response to nerve injury. Peripheral nerve injury can result in a significant increase in the synthesis and subsequent availability of a multitude of extracellularly acting neurotrophic factors, including, but certainly not limited to, NGF (reviewed in Raivich and Makwana, 2007). This enhanced expression of neurotrophins is due in part to the infiltration of macrophages associated with the inflammatory reaction, which secrete cytokines to induce NGF synthesis by Schwann cells and fibroblasts in the lesioned area (Heumann et al., 1987a; Heumann et al., 1987b).

Neurotrophins are synthesized as a precursor form, which undergoes post-translational processing to generate mature forms (Fahnestock et al., 2004; Lee et al., 2001). Neurotrophin activity is thus regulated by the proteases (including furin and other pro-hormone convertases) responsible for the conversion of pro-neurotrophin to its mature form. Inefficient intracellular processing results in the secretion of substantial quantities of the biologically active uncleaved pro-neurotrophin form which preferentially activates p75NTR, the low affinity neurotrophin receptor, and this ligation is associated with the induction of signalling for apoptosis (Lee et al., 2001). Pro-neurotrophin secretion is significantly enhanced following axonal insult or degeneration (Fahnestock et al., 2001; Harrington et al., 2004; Pedraza et al., 2005), and the survival or death of neurons co-expressing both neurotrophin receptors could depend on the relative processing of neurotrophin ligands: increased processing favors Trk-dependent survival, while decreased processing favors p75NTR-mediated death (Lee et al., 2001). The processing, packaging, transport and secretion of neurotrophins are all important post-transcriptional regulators of neurotrophin activity, but the biological responses elicited by



neurotrophins are often specified not only by the ligand itself, but also by the temporal pattern and spatial localization of stimulation (Segal, 2003).

Neurotrophin signalling is broad based, dynamically regulated and context dependent. NGF alone is capable of promoting an exceptionally varied set of responses that requires a highly regulated and widely cascading signal transduction mechanism. The extensive repertoire of potential neurotrophin signalling elements permits minute distinctions depending on the relative expression of downstream effectors, and the complexity is partially explained by the dual high and low affinity receptor paradigm that regulates responses to the NGF family of neurotrophins (Friedman and Greene, 1999; Reichardt, 2006; Sofroniew et al., 2001). In the adult, neurotrophins and their receptors continue to be expressed and dynamically regulated by a variety of cell types (Huang and Reichardt, 2001; Reichardt, 2006; Sofroniew et al., 2001). NGF is further demonstrated to have dual modes of function: signalling locally has been shown to regulate growth cone motility and thus promote neurite extension and steering, while internalization and transport to the cell soma promotes survival and differentiation through actions on gene induction to ensure viability of developing sensory and sympathetic neurons (Huang and Reichardt, 2001; Kaplan and Miller, 2000; Reichardt, 2006; Sofroniew et al., 2001). Neurotrophins are also recognized to promote axonal extension of responsive neurons including CNS axons. Different categories of CNS neurons express neurotrophin receptors and respond in culture to NGF, BDNF and NT-3 stimulation. During postnatal development, many neurons lose absolute dependence on growth factors for acute survival, and thus neurotrophins change primary function from the promotion of survival to the support of process outgrowth and the determination of phenotype (Davies, 1998;

Lewin and Barde, 1996). Mature sensory neurons do not require NGF derived from the periphery for continued survival (Lindsay, 1988; Rich et al., 1984) and exogenous application of NGF, or injury induced upregulation, can often result in inappropriate sprouting and consequent hyperalgesia. In contrast, many sympathetic neurons require NGF both during development and in the adult for continued survival. The molecular signals that control injury response and attempts at repair are just beginning to be untangled, but clearly involve the dynamic regulation of growth factors and their receptors.

### **1.3.2 Neurotrophin Receptors**

Neurotrophin receptors are widely expressed in the PNS and CNS both during development and in the adult. They are present on both pre-synaptic axon terminals and post-synaptic dendrites, and as such neurotrophins can function as target-derived factors influencing developing afferents, but also display anterograde influence to regulate post-synaptic targets (Lessmann et al., 2003). Neurotrophin responsive cells display both low affinity and high affinity binding sites, consistent with the concept that the biological effects of NGF family of neurotrophins are mediated by two structurally distinct receptors (Bothwell, 1995; Kaplan and Miller, 1997). Each neurotrophin binds to a specific member of the Trk family of receptor tyrosine kinases (RTK) with high affinity. NGF binds to TrkA, both BDNF and NT-4/5 interact with TrkB, and NT-3 preferentially binds TrkC. Additionally all of these neurotrophins are able to interact with p75NTR, the low affinity pan-neurotrophin receptor. Neurotrophin responsiveness is dictated by the



relative cell type specific expression of Trk and/or p75NTR, which are frequently, but not always, coexpressed. When coexpressed, Trk and p75NTR exist in a paradoxical relationship and can either collaborate with or inhibit each other's actions to coordinate and modulate neuronal responses to stimulation. As such, receptor activation can result in a wide variety of responses, both acute local effects and alterations in gene expression initiated by retrograde transport (reviewed in Arevalo and Wu, 2006; Hennigan et al., 2007; Huang and Reichardt, 2001; Lewin and Barde, 1996; Reichardt, 2006; Sofroniew et al., 2001).

#### **1.3.2.1 Trk Receptors**

Trk receptors are 140 kDa transmembrane glycosylated protein products encoded by a proto-oncogene, which belong to a family of RTKs. Each Trk contains an extracellular region comprised of immunoglobulin (Ig)-like binding domains. The membrane proximal Ig domain (Ig-2) is the major site of ligand binding, though other extracellular regions have been demonstrated to contribute to ligand binding, either directly or allosterically. A short transmembrane domain spans the membrane once and terminates with a cytoplasmic domain containing sites of tyrosine kinase activity (Huang and Reichardt, 2001; Reichardt, 2006; Sofroniew et al., 2001).

Trk receptors are primarily activated by the mature, and not the pro- form of the neurotrophin gene product (Lee et al., 2001). Ligand binding to the Ig-2 domain of Trk results in receptor homodimerization and subsequent transphosphorylation of specific tyrosine residues on the cytoplasmic tail. Tyrosines at positions 670, 674 and 675 are



within the autoregulatory loop (activation domain) of the tyrosine kinase domain, which controls tyrosine kinase activity (Huang and Reichardt, 2003). Phosphorylation of the activation domain is sufficient to recruit Trk into lipid rafts following ligand engagement, where it is placed in proximity to a concentration of effector molecules (Du et al., 2000; Du et al., 2003). Two tyrosine residues outside of the activation domain form the major sites of endogenous autophosphorylation downstream of receptor activation. With regard to TrkA, the tyrosine at position 490 within the NPXY motif of the juxtamembrane domain, and the tyrosine at position 785 within the YLDIG motif of the carboxy terminus generate docking sites for phosphotyrosine binding (PTB) and Src-homology 2 (SH2) domain containing proteins (Loeb et al., 1994; Obermeier et al., 1993a; Obermeier et al., 1993b; Stephens et al., 1994) to initiate the activation of well-characterized signalling cascades (Figure 1.2) including those involving Ras-MAPK, PLC- $\gamma$ , and PI3K, which will be discussed in detail below.

Different populations of neurons express different Trk receptor isoforms. While the Trk expression dictates neurotrophin responsiveness, Trk family members A, B and C have highly homologous intracellular domains: the phosphorylation sites and downstream signalling cascades are very similar in nature to those described for TrkA above (Atwal et al., 2000; Middlemas et al., 1994; Yuen and Mobley, 1999; Zirrgiebel et al., 1995). Notwithstanding, signalling initiated by different Trk ligations has distinct subtleties important to biological responses. In the case of TrkB which binds two distinct ligands, the signals initiated by each are not identical. NT-4/5 ligation initiates greater activation of MAPK and c-fos than BDNF (Minichiello et al., 1998). During development, TrkA can also be activated by two distinct ligands, as NT-3 exhibits binding promiscuity in the

**Figure 1.2: Schematic illustration of TrkA, the high affinity NGF receptor and its associated signaling pathways.** TrkA ligation results in the phosphorylation of Y674/675 of the activation loop, and phosphorylation of Y490 and Y785. Y490 phosphorylation creates a docking site for Shc, and results in the subsequent activation of the Ras-MAPK cascade. Phosphorylated Y785 interacts with PLC $\gamma$ , which signals downstream to activate several PKC isoforms, as well as the MAPK cascade. TrkA ligation is also associated with the activation of the PI3K-Akt signaling cascade. Adapted from Tucker and Mearow, 2008, *in press*.

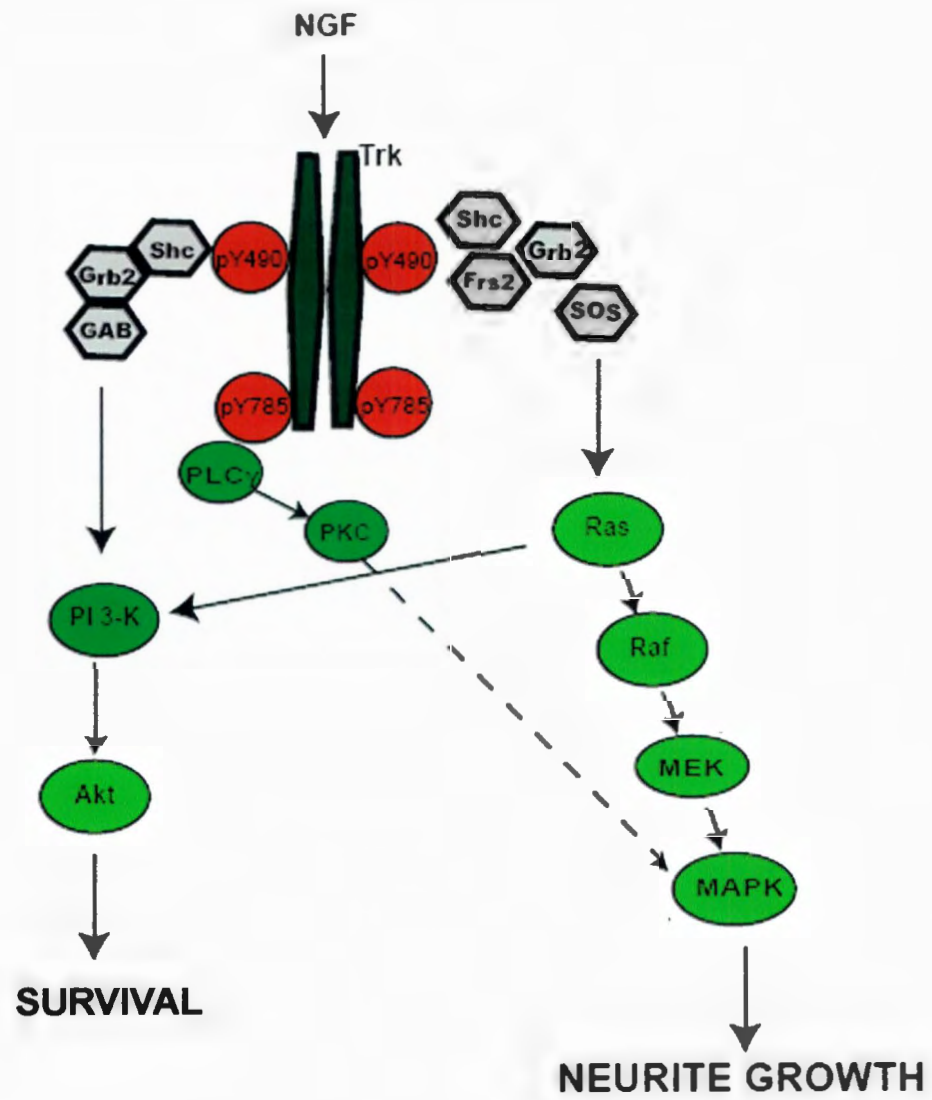


Figure 1.2



absence of p75NTR. NGF and NT-3 both signal through TrkA in developing sympathetic neurons to stimulate proximal and distal extension respectively. Although both neurotrophins use the same signalling mechanisms to promote sympathetic axon growth at two different stages of development, only NGF:TrkA interactions can support the retrograde transport to mediate survival (Belliveau et al., 1997; Glebova and Ginty, 2004; Kuruvilla et al., 2004) thus awarding the target-derived neurotrophin (NGF) exclusive control over the precise number of neurons that ultimately remain to innervate the target (Kuruvilla et al., 2004). Finally, specific subtleties in receptor-ligand interaction direct the subsequent intracellular signalling activities and retrograde transport which allows Trk receptor activation to regulate gene expression and thus participate in a broad range of cellular behaviours including cell survival, proliferation, differentiation, synaptic plasticity, and importantly, remodelling of the cytoskeleton for axonal extension both during development and following nerve injury (Arevalo and Wu, 2006; Huang and Reichardt, 2003).

The interaction of NGF with TrkA at the nerve terminal and subsequent receptor activation has local effects on signalling at axon terminals that affect growth cone motility and guidance, but is also thought to induce the internalization and retrograde transport of the ligand-receptor complex (Beattie et al., 1996; Grimes et al., 1997; Grimes et al., 1996). This has been suggested to occur via formation of an endocytic vesicle, which contains not only catalytically active Trk, but may also contain a variety of signalling intermediates with which Trk interacts including Shc, Rap, ERK1/2 and PLC $\gamma$  (Howe et al., 2001; Wu et al., 2001). For this reason, this structure is referred to as a “signalling endosome”. These vesicles are then transported to the nucleus in a dynamin-

dependent manner (York et al., 2000; Zhang et al., 2000), where they induce nuclear responses to affect gene transcription (Reichardt, 2006).

#### **1.3.2.1.1 Trk-induced Ras-MAPK signalling**

The Ras-MAPK pathway is a consistent and conserved cascade for the transduction of extracellular stimuli, and amplification of associated cellular responses, and results in transcriptional regulation essential to survival and differentiation in many neuronal types (reviewed in Arevalo and Wu, 2006; Huang and Reichardt, 2003; Kaplan and Miller, 2000). It also plays a role in the promotion of neurite outgrowth, though this appears to be cell-type specific. The activity of the Ras-MAPK cascade is necessary and sufficient to induce neurite outgrowth in PC12 cells (Burry, 2001) and plays a key role in sympathetic axonal extension (Glebova and Ginty, 2005), but is not associated with neurite outgrowth of SH-SY5Y neuroblastoma cells and is of minimal importance to adult DRG growth (Kimpinski and Mearow, 2001).

The activation of the TrkA receptor results in transphosphorylation of tyrosine 490 of the cytoplasmic tail, which creates a docking site for PTB and SH2 domain containing proteins (Obermeier et al., 1993b; Stephens et al., 1994). This is the site of interaction with adapter protein, Shc, which becomes activated upon binding and subsequently recruits the growth factor related binding protein (Grb2)-Son of Sevenless (SOS) complex. SOS is a GEF for Ras and thus results in the rapid yet transient activation of Ras (Stephens et al 1994). Activated Ras subsequently promotes the sequential activation of protein kinases Raf, MAPK kinase (MEK) and the MAPKs ERK1

and ERK2 (English et al., 1999). The phosphorylation of ERK can also activate Rsk kinases resulting in the phosphorylation of CREB necessary for transcription of genes that regulate differentiation and survival (Pearson et al., 2001). Furthermore, the phosphorylation of ERK on crucial tyrosine and threonine residues induces its nuclear translocation for the activation of transcription factors (Pearson et al., 2001). ERK1 and 2 activate Elk-1 (Segal, 2003), whose phosphorylation stimulates its interactions that collectively induce c-fos, an early transcriptional event necessary for differentiation (Ginty et al., 1994; Greenberg et al., 1986; Sheng and Greenberg, 1990).

The activation of MAPK via the above detailed mechanism is transient. The prolonged activation of MAPK may require the interaction of Trk with FRS-2, an N-myristoylated protein which is also able to interact with tyrosine 490 of the TrkA cytoplasmic tail (Kao et al., 2001; Meakin et al., 1999). Activated FRS-2 subsequently recruits adapter protein Grb2 and protein phosphatase SH-PTP2, but also Crk which binds and activates Rap1GEF C3G (Meakin et al., 1999), thus resulting in activation of Rap1 and B-Raf, and the prolonged activation of MAPK necessary for differentiation.

Multiple signalling cascades can result in MAPK activation and each has overlapping but distinct targets in the cell, allowing both convergent and divergent signalling cascades to ensure robust and sustained activation of ERK, with the possibility of subtle modulation of temporal dynamics and resultant control of gene transcription and consequent biological responses.

#### **1.3.2.1.2 Trk-induced PLC- $\gamma$ -PKC signalling**



The PLC- $\gamma$ -initiated signalling pathway is a distinct neurotrophin-induced signalling cascade that has the ability to influence growth cone motility and subsequent axon extension in response to extracellular stimuli, exerting both local and transcriptional effects. Trk ligation and dimerization also results in transphosphorylation of tyrosine at position 785 in TrkA cytoplasmic tail, creating a docking site for the direct SH2 domain-mediated interaction of PLC- $\gamma$ 1, which subsequently becomes tyrosine phosphorylated (Stephens et al., 1994). Activated PLC- $\gamma$ 1 can then employ its enzymatic phospholipase activity to catalyze the hydrolysis of membrane phosphatidylinositides, resulting in the release of products inositol 1,4,5 triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> binds specific intracellular receptors to promote the release of Ca<sup>++</sup> from intracellular stores, thereby elevating intracellular Ca<sup>++</sup> concentration which can indirectly activate MAPK and PI3K signalling cascades (Rhee, 2001). DAG promotes the activation of multiple PKC isoforms, including PKC- $\delta$ . PKC- $\delta$  activity in particular is capable of promoting neurite outgrowth of PC12 cells by multiple means, including activation of MEK-ERK cascade (Corbit et al., 1999), apparently acting between Raf and MEK, and signalling via PLC- $\gamma$ -PKC- $\delta$  is reported to control the expression and activity of multiple proteins including transcription factors, growth associated proteins (GAP-43), and ion channels of the TRP family (Klein et al., 2005; Minichiello et al., 2002; Toledo-Aral et al., 1995). As such, PLC- $\gamma$  signalling is involved in the growth response at many levels and is appreciated to have a notable role in synaptic plasticity. Furthermore, PLC- $\gamma$  activity is implicated in chemotropism and regulation of growth cone behaviour, both through the activation of PKC isoforms downstream and through the formation of an intracellular Ca<sup>++</sup> gradient, which is essential for growth cone guidance (Arevalo and Wu, 2006).

#### **1.3.2.1.3 Trk-induced PI3K signalling**

In several neuronal populations, neurotrophin-induced PI3K activity is critical for the ability of the Trk receptor to convey signals for the promotion of cell survival. In fact, PI3K activation and downstream phosphorylation of Akt are demonstrated to be responsible for up to 80% of neurotrophin-induced survival of PC12 cells, as well as cerebellar, sympathetic, sensory, cortical and motor neurons (Bartlett et al., 1997; Crowder and Freeman, 1998; Dudek et al., 1997).

Class I PI3Ks are heterodimers comprised of a regulatory subunit (p85) and a catalytic subunit (p110) which are constitutively associated (Fruman et al., 1998). Trk ligation and subsequent activation can in turn activate PI3K by a variety of different signalling interactions. The phosphorylation of tyrosine at position 490 of the TrkA cytoplasmic tail creates a docking site for Shc (Obermeier et al., 1993b). Activated Shc can subsequently recruit Grb2 and/or Gab1/2. These intermediaries can bind the regulatory subunit of PI3K leading to its activation (Holgado-Madruga et al., 1997). Gab1 is of particular importance as its disruption decreases survival in PC12 cells (Holgado-Madruga et al., 1997). Additionally, PI3K can be stimulated by direct interaction of the catalytic subunit with activated Ras (Downward, 1998), and the inhibition of Ras can impair survival by suppressing PI3K activity (Figure 1.3).

Importantly, the activation of PI3K occurs at the plasma membrane rather than in signalling endosomes. In fact, the inhibition of Trk endocytosis results in increased PI3K

activity (MacInnis and Campenot, 2002; York et al., 2000; Zhang et al., 2000). This membrane localization is important to the downstream function of PI3K. Once activated, PI3K acts as a lipid kinase and generates 3' phosphoinositides: primarily PIP<sub>3</sub>, but also PIP and PIP<sub>2</sub>, all of which display signalling capacity. 3' phospholipids are capable of binding to the PH domain of a variety of downstream effectors, including PDK-1 and Akt, resulting in activation (reviewed in Kaplan and Miller, 2000; Reichardt, 2006). Lipid bound PDK-1 further activates Akt (Alessi et al., 1997). Akt activity subsequently serves as a hub for the activation of a plethora of cascades with multiple effects including, but not limited to, the prevention of apoptosis and the concomitant promotion of survival. In this regard, Akt substrates include BAD, a proapoptotic protein which is sequestered upon phosphorylation and thereby rendered ineffective (Datta et al., 1997), and members of the Forkhead transcription factors (FKHRL1) which are also sequestered upon phosphorylation to prevent the transcription of proapoptotic genes (Brunet et al., 2001; Zheng et al., 2002). Phosphorylated Akt can also activate NFκB via phosphorylation of its inhibitor IκB and targeting it for degradation (Foehr et al., 2000; Hamanoue et al., 1999; Wooten et al., 2001), thus releasing NFκB and consequently promoting the transcription of pro-survival genes.

The activation of PI3K has also been implicated in the regulation of neurite outgrowth, guidance and differentiation in some populations of neurons. 3' phosphoinositides generated by PI3K activation are able to recruit GEFs for Cdc42 and Rac to the plasma membrane thereby exerting regulatory control over cytoskeletal organization and resulting in the promotion of lamellipodial and filopodial formation (Yuan et al., 2003). Furthermore, downstream phosphorylation of Akt can subsequently



**Figure 1.3 Schematic illustration of the Trk-induced activation of the PI3K-Akt signaling cascade.** TrkA ligation results in the activation of PI3K, which increases the concentration of 3' phosphatidylinositols, resulting in the activation of Akt. Akt subsequently activates a variety of downstream signaling pathways which impact survival and growth in many systems. PTEN negatively regulates the activity of PI3K, acting to decrease the concentration of 3'-phosphatidylinositols, and thereby impairing the activation of Akt. Adapted from Penninger and Woodgett, Science, 2001 Dec. 294(5549): 2116-2118.

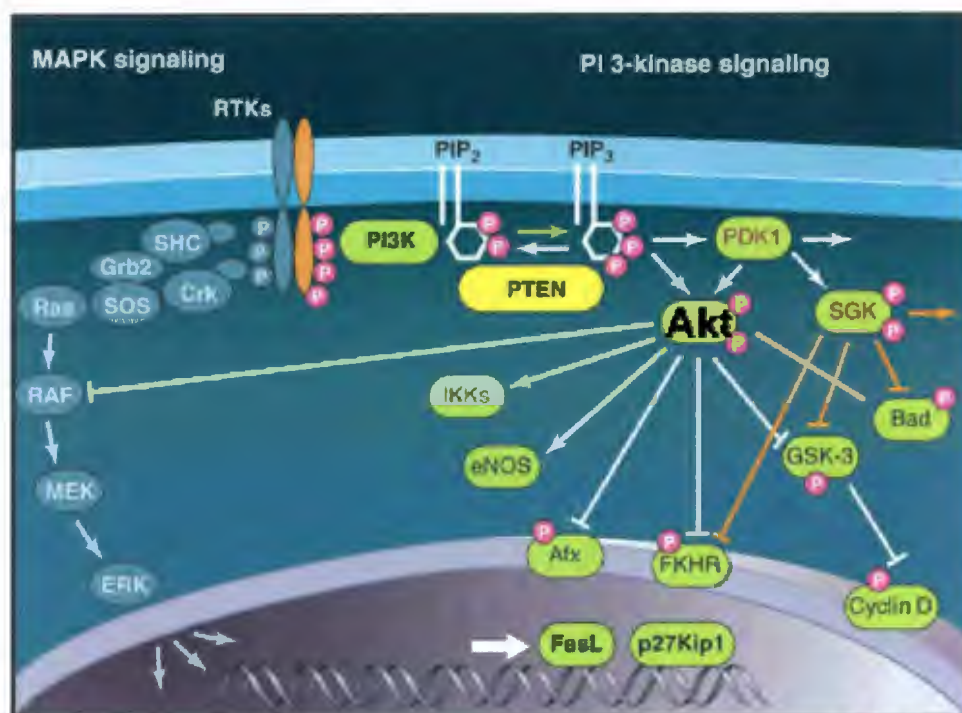


Figure 1.3

phosphorylate and inactivate GSK3 $\beta$ , an event known to promote neurite outgrowth, at least partially through regulation of microtubule binding protein, APC (Chin et al., 2005; Filipenko et al., 2005; Zhou et al., 2004).

The primary endogenous antagonist of PI3K signalling is a phosphatase known as phosphatase and tensin homolog deleted on chromosome ten (PTEN) (Figure 1.3). PTEN possesses a large binding pocket and is thus able to accommodate the bulky 3'phosphoinositides, resulting in their dephosphorylation, which subsequently negatively regulates the activation of Akt and its associated survival pathways (Salmena et al., 2008). In addition to its lipid phosphatase activities, PTEN also possesses protein phosphatase activities *in vitro*, although the purpose *in vivo* remains largely unknown (Yamada and Araki, 2001). The activation of PI3K-Akt signalling is likely to mediate survival and growth on a number of levels depending on cell type and target availability, and thus its regulation is central to the development of a variety of neuronal populations. Nonetheless, PTEN is a versatile protein that exhibits biological properties in addition to PI3K antagonism. Genetic deletion studies have demonstrated a crucial role for PTEN in normal brain development. Specifically, PTEN function appears to be vital to the regulation of proper neuronal size, migration, polarity, differentiation, genomic stability and process morphology (reviewed in Chang et al., 2007; Salmena et al., 2008; van Diepen and Eickholt, 2008). Its deletion or mutation is therefore associated with multiple defects in neural development including disorganized patterning, hypertrophy (Backman et al., 2001; Groszer et al., 2001; Kwon et al., 2001; Li et al., 2003; Marino et al., 2002; Nayeem et al., 2007) and the development of numerous tumour associated syndromes (Li and Sun, 1998; Liaw et al., 1997). Alterations in PTEN function have also proven crucial



for responses that require dynamic regulation of the neuronal cytoskeleton (Arevalo and Rodriguez-Tebar, 2006; Chadborn et al., 2006), through mechanisms that appear to be PI3K independent (Mahimainathan and Choudhury, 2004; Tamura et al., 1998). PTEN shares significant sequence homology with cytoskeletal components tensin and auxillin (Li et al., 1997), suggesting that its targets may be cytoskeletal (Myers and Tonks, 1997), and PTEN is proposed to play a role in neurite outgrowth potentially via regulation of phosphorylation dependent interactions between tau and microtubules (Zhang et al., 2006a; Zhang et al., 2006b). Thus the functional relevance of PTEN extends beyond suppression of tumours and antagonism of PI3K. This phosphatase is emerging as a key regulator of development and normal physiology, as well as pathological states in both the developing and adult nervous systems (Chang et al., 2007; Li et al., 2003; van Diepen and Eickholt, 2008).

#### **1.3.2.2 p75NTR Receptor**

p75NTR was the first identified receptor for NGF (Johnson et al., 1986) and was subsequently shown to bind each neurotrophin with similar low affinity (Rodriguez-Tebar et al., 1990); neurotrophins bind p75NTR with approximately 1000 fold lower affinity than they bind to their cognate Trk receptors. Despite the ability of p75NTR to bind all neurotrophins of the NGF family, the binding kinetics of each interaction differs in terms of rate constant and spatial arrangements of positively charged residues, thus providing p75NTR the ability to discriminate among ligands (Rodriguez-Tebar et al., 1990; Rodriguez-Tebar et al., 1992; Ryden et al., 1995).

p75NTR is a 399 amino acid, Type 1 transmembrane protein that is a member of the tumor necrosis factor receptor (TNFR) superfamily. While p75NTR shares several specifically conserved TNFR characteristics including the extracellular domain cysteine rich repeats (Yan and Chao, 1991) and the intracellular death domain (Liepinsh et al., 1997), it also displays significant deviation from the standard TNFR activity. p75NTR demonstrates a propensity to dimerize rather than trimerize like the majority of TNFRs, and binds NGF with a 2:1 stoichiometry unlike other TNFRs (He and Garcia, 2004).

The p75NTR promoter lacks consensus TATA or CAAT sequences, but instead displays multiple GC-rich sequences which represent Sp1 binding motifs (Sehgal et al., 1988). The resultant gene product displays a distinct lack of catalytic motifs. The ability of p75NTR to transduce signals is instead largely dependent upon two potential mechanisms: the availability of co-receptors and the subsequent recruitment of intracellular signalling partners (reviewed in Reichardt, 2006; Roux and Barker, 2002), and a cleavage cascade. In this regard, p75NTR undergoes a series of sequential cleavages: proteolytic shedding of the extracellular domain is followed by regulated intramembrane proteolysis (RIP). This process is dependent upon  $\alpha$ - and  $\gamma$ -secretases, and results in the release of an intracellular domain fragment, which is thought to possess signalling capabilities (DiStefano and Johnson, 1988; Jung et al., 2003). In support of this, a cleavage-resistant mutant version of p75NTR demonstrated altered ability to interact with other proteins and activate NF $\kappa$ B (Zampieri and Chao, 2006).

p75NTR is highly expressed in the developing nervous system by a wide variety of neuronal populations in both the CNS and PNS, where it is thought to contribute to appropriate growth and development of the nervous system. Neural crest cells express



p75NTR as they migrate to sites of ganglia formation, and levels increase as they undergo differentiation (Heuer et al., 1990). Sympathetic and most sensory neurons, cerebellar neurons, cholinergic neurons of the basal forebrain, retinal neurons, hippocampal neurons and motor neurons of the spinal cord all express p75NTR during the period of developmental axon extension (Buck et al., 1987; Ernfors et al., 1988; Large et al., 1989; von Bartheld et al., 1991; Yan and Johnson, 1988). Typically, p75NTR expression is dramatically reduced following target innervation; expression becomes more restricted in the adult nervous system where it is often downregulated to undetectable levels (1% of embryonic levels) (Heumann et al., 1987b) but it is notably retained in the adult sympathetic and sensory populations as well as in basal forebrain cholinergic neurons (Ruit et al., 1990; Verge et al., 1989).

p75NTR expression can also be robustly induced by injury in a wide variety of cell types, and is associated with multiple pathological or chronic degenerative diseases, including seizure (Roux et al., 1999), spinal cord injury (Dubreuil et al., 2003), ischemia (Greferath et al., 2002), multiple sclerosis, diabetic neuropathy, Alzheimer's disease (Yaar et al., 1997), and is a prognostic marker on multiple tumour types (Descamps et al., 2001). p75NTR expression is closely correlated with degenerative changes and apoptosis observed after neuronal trauma, and can be experimentally induced in the CNS by kainic acid (Hennigan et al., 2007). It is unclear whether p75NTR induction plays a role in the progression of these diseases, or is part of an attempt at regeneration.

While the engagement of Trk almost certainly elicits growth, survival, and differentiation responses, the functional role of p75NTR is far less clearly understood. Genetic deletion studies have been hindered by the continued expression of alternatively



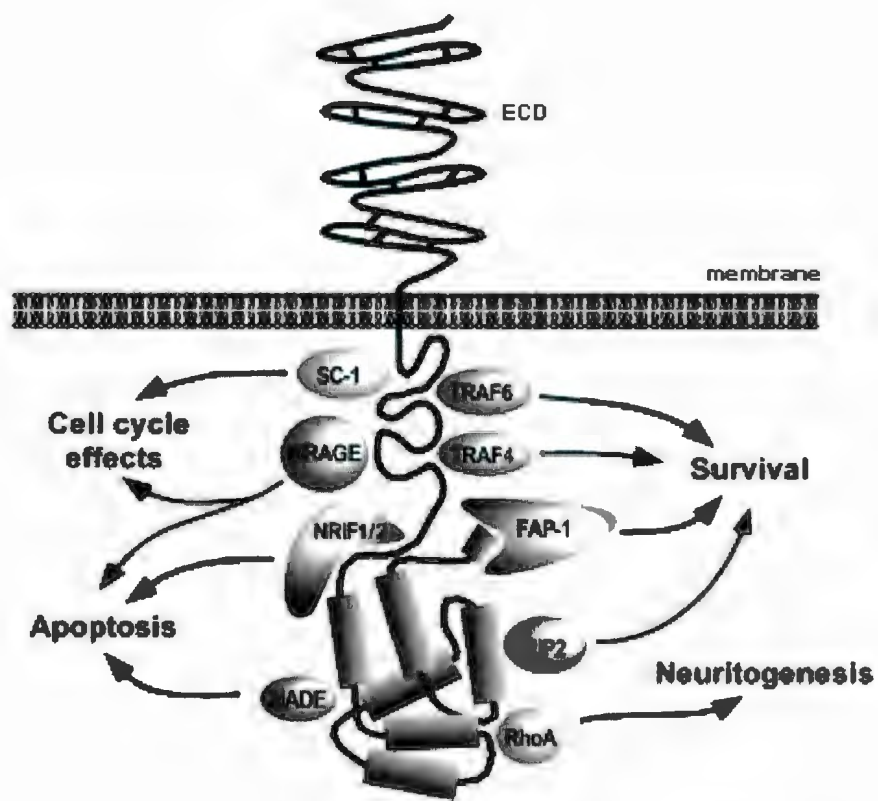
spliced and truncated versions thus confounding results (Paul et al., 2004; von Schack et al., 2001). Nevertheless, the functions attributed to p75NTR are diverse, complex and somewhat paradoxical: p75NTR activity has been linked to the promotion of both survival and apoptosis, the promotion and inhibition of neurite outgrowth, and the enhancement of both proliferation and differentiation (reviewed in Arevalo and Wu, 2006; Roux and Barker, 2002) (Figure 1.4). The physiological outcome of p75NTR activation appears to depend upon ligand identity, co-receptor availability and intracellular interactions. Cell type and developmental stage may further influence biological responses mediated by p75NTR associated signalling, both p75NTR autonomous cascades and Trk-dependent signalling.

#### **1.3.2.2.1 p75NTR Autonomous Signaling**

##### **1.3.2.2.1.1 p75NTR Signalling for Apoptosis**

While the exact purpose of this receptor has been a matter of considerable controversy for many years, it has been well-characterized for its role in the promotion of apoptosis in the absence of Trk receptor co-expression. In the absence of concurrent Trk activation, neurotrophin ligation of p75NTR can facilitate apoptosis (Rabizadeh et al., 1993). The addition of BDNF to cultures of sympathetic neurons, which lack TrkB expression, causes an apoptotic response (Bamji et al., 1998) that can be abrogated by the introduction of Trk, (Davey and Davies, 1998; Yoon et al., 1998). Furthermore,

**Figure 1.4: Schematic illustration of selected p75NTR cytoplasmic interactions and outcomes.** p75NTR signals via the recruitment of various intracellular signaling proteins and adapters which allows p75NTR signaling to result in a variety of different outcomes depending upon context. Adapted from Roux and Barker, *Progress in Neurobiology*, 2002 June 67(3): 203-233.





neurotrophin-dependent apoptosis occurs in TrkA knockout mice, thereby suggesting this outcome to be the result of p75NTR autonomous signalling (Majdan et al., 2001).

The presence of p75NTR is also essential for the induction of apoptosis that ensues following growth factor withdrawal or NGF deprivation (Barrett, 2000; Barrett and Georgiou, 1996). A decrease in p75NTR expression correlates with decreased or delayed apoptosis (Bamji et al., 1998), while an increase in p75NTR expression accelerates apoptosis, and is associated with a decreased number of cortical, sensory and sympathetic neurons (Majdan et al., 1997). Injury-induced expression of p75NTR also correlates with enhanced apoptosis, and the use of p75NTR antisense oligonucleotides can rescue axotomized neurons from death (Cheema et al., 1996).

There are multiple means established by which p75NTR is recognized to induce apoptosis, and the majority of them depend upon the orchestration of several downstream interacting proteins. The cytoplasmic tail of p75NTR is appreciated to interact with a variety of adapters and transcription repressors which have been demonstrated to disrupt the cell cycle and promote apoptosis (reviewed in Roux and Barker, 2002). These include NRIF, NUAGE, TRAF-6, NADE and SC-1. NUAGE is recruited to the plasma membrane upon NGF stimulation and interferes with the association of p75NTR with Trk (Salehi et al., 2000), while NADE associates directly with the p75NTR intracellular death domain in an NGF-dependent fashion and promotes the activation of caspases for the induction of apoptosis. Dominant negative caspase-9 is demonstrated to attenuate p75NTR-induced apoptosis (Wang et al., 2001). JNK signalling is also recognized to contribute to caspase-9 activation (Bruckner et al., 2001), and adapter TRAF-6 links p75NTR to the activation of the JNK signalling cascade (Wajant et al., 2001; Yoon et al.,

1998) which results in the activation of p53 (Aloyz et al., 1998). p53 has multiple targets, including proapoptotic Bax and the induction of FasL expression (Le-Niculescu et al., 1999). JNK is strongly activated following p75NTR ligation, and following NGF withdrawal (Aloyz et al., 1998), and this can be prevented by genetic deletion of TRAF-6 or NRIF (Linggi et al., 2005; Yeiser et al., 2004), or by the concurrent activation of Trk which silences JNK-p53 death via Ras and PI3K activity for the repression of apoptosis (reviewed by Kaplan and Miller, 2000).

Ligand binding to p75NTR is also known to activate sphingomyelinase activity in a trk-independent fashion to initiate sphingomyelin hydrolysis and the generation of ceramide (Dobrowsky et al., 1995). Ceramide is a structural component of the cell membrane, but it also possesses second messenger capabilities that can induce apoptotic effects (reviewed by Blochl and Blochl, 2007) in different types through modulation of many signalling cascades including ERK, JNK, NF $\kappa$ B (reviewed by Huang and Reichardt, 2001), and may directly inhibit PI3K activity (Zhou et al., 1998).

Finally, as previously mentioned, p75NTR serves as the preferred receptor for the pro-form of neurotrophins, which bind p75NTR with higher affinity and induce apoptosis at lower concentrations (Lee et al., 2001). The induction of apoptosis by pro-neurotrophins requires the interaction of p75NTR with co-receptor sortilin, a Vps10p domain containing protein (Nykjaer et al., 2004). The ability of p75NTR to induce rapid and appropriate apoptosis during development allows for the elimination of neurons unsuccessful in obtaining appropriate trophic support and thus regulates innervation density and patterning (reviewed in Huang and Reichardt, 2001). The apoptotic function



of p75NTR could also be critical following neuronal injury or degeneration (Ferri et al., 1998; Roux et al., 1999).

#### **1.3.2.2.1.2 p75NTR Signalling for Survival**

Paradoxically, signalling via p75NTR is also implicated in the promotion of survival, primarily via the ligand-dependent activation of NF $\kappa$ B. An NGF-induced interaction between p75NTR and TRAF-6 results in the sequential phosphorylation of I $\kappa$ K and I $\kappa$ B resulting in the liberation of NF $\kappa$ B, which translocates to the nucleus to trigger prosurvival expression changes (Hamanoue et al., 1999; Middleton et al., 2000).

Furthermore, NGF exposure is able to promote the survival of cortical neurons from excitotoxicity, which must be dependent upon p75NTR, since these neurons do not express TrkA (Kume et al., 2000; Shimohama et al., 1993), and a mutant form of NGF that binds only p75NTR can inhibit apoptosis induced by serum deprivation (Hughes et al., 2001) potentially via the activation of Akt (Roux et al., 2001). A role for p75NTR in the promotion of survival is supported by studies of the p75NTR exon IV knockout mouse, which presents with a severe loss of peripheral sensory neurons (von Schack et al., 2001).

#### **1.3.2.2.1.3 p75NTR Signalling for Growth**

Another major biological function of p75NTR is to dramatically influence growth: p75NTR can promote growth in collaboration with Trk (see section 1.3.2.3), but can also inhibit growth acting autonomously using signalling interactions distinct from those



activated by Trk (Hempstead, 2002). p75NTR knockout mice exhibit slower extension of axons (Bentley and Lee, 2000) and reduced outgrowth (Yamashita et al., 1999) of both motor and sensory neurons, but the elimination of p75NTR also led to robust sprouting of adult sympathetic nerve fibres on myelin and resulted in cholinergic hyperinnervation of the hippocampus (Yeo et al., 1997). Additionally, selective activation of p75NTR, by stimulating NGF-dependent DRG neurons with BDNF for example, impairs neurite outgrowth (Kimpinski et al., 1999).

p75NTR constitutively activates Rho, a small GTPase known to potently inhibit axonal outgrowth, by functioning as a displacement factor to facilitate the release of Rho from Rho-GDI (Yamashita and Tohyama, 2003; Yamashita et al., 1999). In fact, the overexpression of p75NTR is sufficient to activate endogenous Rho in a variety of neuronal and non-neuronal cell types, and this activation inhibits the cytoskeletal remodelling necessary for axonal extension. This growth inhibition is reversed upon neurotrophin binding to p75NTR, which abrogates the p75NTR-dependent activation of Rho (Yamashita et al., 1999), thus permitting neurite outgrowth.

p75NTR interaction with co-receptor NgR and subsequent transduction of signals from myelin associated inhibitory proteins also results in the activation of Rho and the associated inhibition of neurite outgrowth (Fournier et al., 2003; Schweigreiter et al., 2004; Winton et al., 2002). The modulation of Rho activity is critical to p75NTR influence over neurite outgrowth, and this modulation is dependent upon the proportion of unliganded versus liganded p75NTR, as well as the presence of inhibitory ligands notably abundant after injury.

### 1.3.2.3 Trk-p75NTR Interaction

While p75NTR clearly possesses autonomous signalling abilities, it can also signal collaboratively with Trk receptors to enhance neurite outgrowth through a variety of mechanisms. The assembly of multiprotein complexes including receptors and constituents facilitates the physical interaction between p75NTR and Trk receptors. These complexes can include caveolin, which provides a platform for interaction (Bilderback et al., 1999), ARMS, and p62-TRAF-6-IRAK, which associate to form a scaffold for interactions (Wooten et al., 2001) and recruit E2 ubiquitin ligase to facilitate receptor internalization (Geetha et al., 2005).

p75NTR is capable of modulating Trk function on a number of levels. p75NTR collaborates with Trk to promote high affinity neurotrophin binding by assisting in the formation of high affinity binding sites (Hempstead et al., 1991), or acting as a surface reservoir to concentrate the ligands locally and present them to Trk in a favourable binding conformation to increase the rate of association of NGF with Trk. In support of this hypothesis, the disruption of NGF binding to p75NTR impairs the ability of NGF to activate Trk (Barker and Shooter, 1994; Clary and Reichardt, 1994; Lachance et al., 1997; Verdi et al., 1994) and a mutant NGF that binds only Trk is far less efficient at inducing biological responses (Barker and Shooter, 1994; Ryden et al., 1997). p75NTR may also influence Trk allosterically thereby conferring high affinity binding, as the transmembrane and intracellular domains of p75NTR are necessary to mediate this interaction (Esposito et al., 2001).



p75NTR is able to modulate both the affinity and the selectivity of TrkA ligation, potentiating activation in low ligand situations, and inhibiting activation of TrkA by NT-3 (Hantzopoulos et al., 1994; Verdi et al., 1994). It is also reported to enhance the phosphorylation of Trk (MacPhee and Barker, 1997) and its downstream signalling intermediates, including Shc (Epa et al., 2004) thereby enhancing Trk activity and promoting neurite outgrowth. Furthermore p75NTR is reported to influence the ubiquitination of Trk (Arevalo et al., 2006; Geetha et al., 2005; Makkerh et al., 2005). Suppression of ubiquitination delays the internalization and degradation of Trk to prolong signalling. Alternatively, p75NTR has also been reported to promote Trk endocytosis and retrograde transport via recruitment of E2-E3 ubiquitin ligases, such that internalized Trk can promote intracellular signalling reactions.

While p75NTR clearly enhances the function of TrkA, this crosstalk is bidirectional, as the function and activity of p75NTR is dependent upon the activation status of Trk (Bamji et al., 1998; Davey and Davies, 1998). Trk-mediated signalling can actively suppress the proapoptotic signals elicited by p75NTR ligation. In the absence of Trk, neurotrophins are far more effective at inducing apoptosis (reviewed in Huang and Reichardt, 2003) as both the Ras-MAPK and PI3K signalling cascades can inhibit the activation of JNK-p53 resulting in the repression of apoptosis (Mazzoni et al., 1999). This ensures that p75NTR ligation promotes the efficacy of Trk signalling when both receptors are activated. Trk is also reported to suppress ceramide production (Dobrowsky et al., 1995), but does not exert global inhibition over all p75NTR autonomous signalling cascades. Trk does not inhibit NF $\kappa$ B activation (Yoon et al., 1998) and this prosurvival cascade actually represents a key point of collaboration between Trk and p75NTR



(Hamanoue et al., 1999) that provides a synergistic contribution to survival. The two receptors may also synergistically promote survival through activation of Akt (Kaplan and Miller, 2000).

### **1.3.3 The Role of the Extracellular Matrix in Promoting Regenerative Growth**

Like normal nervous system development, axonal regeneration after injury relies on appropriate adhesion to a positive and substantiating substrate and the presence of extrinsic informative cues; both functions that can be fulfilled by molecules that comprise the ECM (reviewed in Luckenbill-Edds, 1997). The composition of the ECM, or basement membrane, is dynamic since the individual protein components secreted and remodelled by resident cells are regulated temporally and spatially throughout development to create subtle distinctions in composition that govern axon pathfinding mechanisms by establishing boundaries between permissive and non-permissive areas, while attractive and repulsive cues orient the movement of the growth cone at the tip of the leading process for the correct positioning of neurons (Giancotti and Ruoslahti, 1999; Lemons and Condic, 2008; Porcionatto, 2006). In fact, the classical function of the ECM is simply to provide an adhesive substrate for axon growth, but is further appreciated for its ability to initiate signalling cascades in a receptor-dependent manner (Miranti and Brugge, 2002).

As the identity of insoluble protein components that comprise the ECM network were individually established, it was discovered that a tripeptide motif (RGD) was common to the majority of ECM proteins and could mediate adhesive interactions with

the cell surface via receptors known as integrins (Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1986). Successful regeneration of the PNS is at least partially attributable to the PNS ECM, which is rich in LN that persists to adulthood (Martin and Timpl, 1987). In contrast, the adult CNS lacks permissive ECM proteins, potentially contributing to the inability of adult CNS axons to regrow. The increased expression of adhesion molecules following axotomy is considered to be one of the driving forces permitting the extension of the elongating axon (Ekstrom et al., 2003; Kloss et al., 1999; Voegelzang et al., 2001; Wallquist et al., 2004; Werner et al., 1998; Werner et al., 2000), and the interaction of the growth cone with the extracellular substrate is critical for regeneration as a large part of regenerative growth depends on molecules that regulate surface-cytoskeletal interaction to modify actin polymerization, organization and disassembly (Baas and Ahmad, 2001; Bouquet et al., 2004; Ellezam et al., 2002; Schmidt et al., 1995; Zhang et al., 2003).

#### **1.3.3.1 Laminin**

Laminins (LN) represent a major component of the basal lamina of the PNS (Martin and Timpl, 1987), and comprise an important family of ECM glycoproteins that bind integrin receptors (reviewed in Luckenbill-Edds, 1997; Mecham, 1991). LN is composed of three polypeptide chains, A, B1 and B2 (also known as  $\alpha$ ,  $\beta$  and  $\gamma$  subunits) connected with disulphide linkages to form a cross-shaped molecule capable of self-assembling to form polymeric sheets (Timpl and Brown, 1996). Multiple LN isoforms (denoted LN1-15) are regionally distributed (Colognato et al., 2005), and vary in



combination of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits. LN-1 is a critical isoform for nervous system development and is abundantly expressed in the PNS, synthesized and secreted into the extracellular environment by resident cells, including Schwann cells, where it interacts with receptors on the cell surface and thereby orchestrates changes in cell behaviour (Luckenbill-Edds, 1997).

Since LN is critical for the formation of the basal lamina, and thus essential to development, genetic ablation of LN has been unable to provide information regarding its postnatal functions (Bonner and O'Connor, 2001). Nonetheless, each specific domain of the LN molecule is recognized to participate in distinct cellular functions, including the promotion of cell adhesion, neurite outgrowth, migration and interaction with other glycoproteins (Powell and Kleinman, 1997; Tashiro et al., 1989), and the function of LN in general appears to change depending on the developmental stage, shifting from directing neural crest cell migration (Previtali et al., 2001; Tarone et al., 2000) to promoting neurite outgrowth and guidance (Forrester and Garriga, 1997; Garcia-Alonso et al., 1996).

In the PNS, LN-1 is a predominant mediator of neurite outgrowth influencing the behaviour of the growth cone by a variety of means (Luckenbill-Edds, 1997). Besides providing an adhesive substrate for growth, LN is further implicated as an attractive guidance cue (Kuhn et al., 1995) itself, and is demonstrated to influence neuronal response to other guidance cues by suppressing intracellular cAMP levels (Hopker et al., 1999).

LN expression is dramatically upregulated following nerve injury (Doyu et al., 1993; Kuecherer-Ehret et al., 1990; Wallquist et al., 2002), and is associated with the



permissive Bands of Bungers (Fawcett and Keynes, 1990). Furthermore, genetic ablation of the LN $\gamma$ 1 subunit impairs regenerative growth (Chen and Strickland, 2003), as does the use of a LN-specific antibody (Wang et al., 1992). LN is also a strong promoter of neurite outgrowth *in vitro* (reviewed in Luckenbill-Edds, 1997). Collectively, these studies indicate a role for LN in axonal regeneration following nerve injury, which could entail supporting survival and migration of Schwann cells and serving as a substrate for axon extension.

#### **1.3.3.2 Integrins**

The cell surface receptors that bind the RGD motif of ECM proteins are a family of proteins known as integrins, named for their ability to integrate extracellular cues with intracellular cytoskeletal changes (Giancotti and Ruoslahti, 1999). Integrins are well recognized for structural roles facilitating adhesion and forming connections between the ECM on the exterior of the cell and the cytoskeleton on the interior of the cell to establish tissue architecture and regulate cell shape, but they are further appreciated for their role in bidirectional transduction of signals, and thus are central to a wide range of biological processes, including proliferation, apoptosis, motility, differentiation, synaptogenesis and axonal extension (Coppolino and Dedhar, 2000; Lemons and Condic, 2008; Schlaepfer and Hunter, 1998). As such, integrin signalling is understood to participate in both the development of the nervous system and axonal regeneration following nerve injury.

Integrins are heterodimeric transmembrane receptors comprised of non-covalently associated  $\alpha$  and  $\beta$  subunits selected from at least 19 $\alpha$  and 8 $\beta$  chains to form more than 24

unique combinations (Alam et al., 2007). The  $\alpha$  and  $\beta$  chains have large extracellular domains that together form the ligand binding domain and each subunit contributes to the ligand selectivity of the receptor. The subunits possess a single transmembrane domain and a short cytoplasmic C-terminus of variable length, but typically short and devoid of catalytic motifs (Giancotti and Ruoslahti, 1999; Lemons and Condic, 2008). Most integrin heterodimers can function as a receptor for multiple ECM components, there is generally more than one integrin pair capable of recognizing any given ligand (Table 1), and many integrins recognize the RGD sequence common to multiple ECM ligands (Hynes, 1992; Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1986). Despite this considerable overlap, integrins are capable of distinguishing specific ligands and can generate both common and specific signals to yield specific biological outcomes (Kumar, 1998).

Integrin receptors are widely expressed in all cell types, including neurons (Lemons and Condic, 2008), but expression is cell-type specific (Kumar, 1998). In neuronal cells the regulation of integrin surface expression is a function of both the amount of available ligand and the specific composition of the ECM (Condic and Letourneau, 1997; Strachan and Condic, 2004). Additionally, a variety of integrin subunits are upregulated following axotomy (Ekstrom et al., 2003; Kloss et al., 1999; Voglezang et al., 2001; Wallquist et al., 2004; Werner et al., 2000). Notably, injury can induce expression of LN-binding integrins in both cell bodies and regenerating axons (Hammarberg et al., 2000), and this increased integrin expression is correlated with successful regeneration of the PNS (Lemons and Condic, 2008). This suggests a role for integrins in successful regenerative growth that warrants further investigation.

**Table 1: Integrins and their Ligands.** Recognized integrin  $\alpha/\beta$  combinations and their ligand interactions are presented here. The specific  $\alpha/\beta$  interactions determine the functional specificity of the receptor complexes. Note that multiple combinations recognize the same ligand, and each  $\alpha/\beta$  combination can recognize multiple ligands. Table adapted from Alam et al (2007) J. Cell. Physiol. 213: 649-653, Takada et al (2007) Genome Biol. 8(215):211-215.



<b>β Subunit</b>	<b>α Subunit</b>	<b>Ligand</b>
β1	α1	Laminin, collagen
	α2	Laminin, collagen, thrombospondin, E-cadherin, tenascin
	α3	Laminin, collagen, fibronectin, entactin, thrombospondin, uPAR
	α4	Fibronectin, VCAM-1, osteopontin, ADAM, ICAM, MAdCAM-1, thrombospondin, Lu/BCAM, CD14, JAM-2, uPAR
	α5	Fibronectin, L1, osteopontin, fibrillin, thrombospondin, ADAM, NOV
	α6	Laminin, thrombospondin, Cyr61, ADAM, uPAR
	α7	Laminin
	α8	Fibronectin, tenascin, nephronectin, vitronectin, osteopontin, TGF-β-LAP
	α9	Tenascin, VCAM-1, osteopontin, uPAR, plasmin, angiostatin, ADAM, VEGF
	α10	Collagen, laminin
	α11	Collagen
β2	αv	Fibronectin, osteopontin, TGF-β-LAP, L1
	α <sub>L</sub>	ICAM
	α <sub>M</sub>	iC3b, fibrinogen, factor X, ICAM, heparin
	α <sub>X</sub>	iC3b, fibrinogen, collagen, ICAM, heparin
β3	α <sub>D</sub>	ICAM, VCAM-1, fibrinogen, fibronectin, vitronectin, Cyr61, plasminogen
	α <sub>II</sub>	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin, disintegrin, osteopontin, Cyr61, ICAM, L1
	αv	Vitronectin, fibrinogen, fibronectin, von Willebrand factor, thrombospondin, fibrillin, tenascin, PECAM-1, BSP, ADAM, ICAM, FAF-2, uPA, angiostatin, TGF-β-LAP, Del-1, L1, MMP, osteopontin, cardiotoxin, uPAR, plasmin, Cyr61, tumstatin, NOV
β4	α6	Laminin
β5	αv	Vitronectin, osteopontin, fibronectin, TGF-β-LAP, NOV, BSP, MGF-E8
β6	αv	Fibronectin, tenascin, vitronectin, TGF-β-LAP, osteopontin, ADAM
β7	α4, α <sub>IEL</sub>	Fibronectin, VCAM, MAdCAM-1, osteopontin
	αE	E-cadherin
β8	αv	Vitronectin, laminin, TGF-β-LAP

#### 1.3.3.2.1 Role of Integrins in Growth

The process of axon extension is contingent upon the protrusion and motility of the growth cone at the leading edge (Huber et al., 2003). Neuronal attachment to the ECM is typically sufficient to induce the formation of filopodia which probe the environment. Integrins concentrated at the tips of the filopodia initiate points of adhesion which allow for the organization of the actin rich lamellipodium as cells spread on the ECM (Giancotti and Ruoslahti, 1999) and regulates growth cone motility. Because of the intimate connection between integrins and actin, integrins serve as a mechanical link between the ECM and cytoskeletal organization, but they also mediate the activation of a remarkable number of classical signalling pathways inside the cell (Schlaepfer and Hunter, 1998).

Integrin activation results in the suppression of cAMP (Lemons and Condic, 2006). As previously noted, enhancing cAMP signalling can promote regenerative growth (Cai et al., 2001; Qiu et al., 2002a; Qiu et al., 2002b), but this is accurate only when integrin activation is low (Lemons and Condic, 2006). Integrin activation combined with elevated cAMP results in growth cone collapse, likely as a result of modulation of Rho GTPases, Rac and Rho. Both cAMP and integrins influence the activity of Rho GTPases (Lemons and Condic, 2006; Ridley, 2000; Schwartz and Shattil, 2000) which in turn, regulate the actin cytoskeleton. Growth cone motility is thus regulated by a balance of integrin activation, cAMP and Rho GTPase activation. Rac activity in particular must be balanced, as both dominant negative and constitutively active forms of Rac suppress neurite extension (Kuhn et al., 1998; Luo et al., 1994).

An array of approaches has revealed the importance of integrins in promoting neurite outgrowth. Genetic deletion of the  $\alpha 7$  subunit (which recognizes LN) is associated with significantly impaired regeneration after facial nerve axotomy (Werner et al., 2000), although these mice upregulate  $\beta 1$  after injury thereby promoting central axonal sprouting (Werner et al., 1998). Conversely the overexpression of integrin subunits is sufficient to restore the regenerative capacity of adult sensory neurons making it comparable to the embryonic situation (Condic, 2001). Additionally, forced integrin expression and the provision of a complimentary ligand can permit extending axons to overcome inhibitory environmental elements including CSPGs and myelin associated components (Condic et al., 1999). As further supporting evidence, the marked induction of LN-binding integrins results after axotomy of the peripheral branch of the DRG axon, but not after injury to the central branch of the DRG axon, thus correlating with regenerative success (Wallquist et al., 2004). This discrepancy can be overcome by a preconditioning lesion, which elevates expression of  $\alpha 5$  and  $\alpha 7$ , subsequently promoting growth cone motility and enhancing growth (Ekstrom et al., 2003; Gardiner et al., 2007). The ability of a neuron to regulate surface expression of integrin receptors significantly contributes to the ability of the growth cone to maintain an intermediate level of attachment and constant motility over widely varying amounts of LN and suboptimal substrata (Condic, 2001; Condic and Letourneau, 1997; Condic et al., 1999; Lemons et al., 2005). In fact, neonatal growth cones can regenerate in CSPG-rich environments by elevating integrin surface expression. Adult neurons do not adapt spontaneously but forced integrin overexpression can abrogate inhibition induced by the environment (reviewed in Lemons and Condic, 2008). Also contributing to this finite control is the



availability of extracellular ligand, and the regulation of the functional state of the integrin; that is, the ability of the intracellular state of the cell to modulate integrin function (Takagi et al., 2002).

#### **1.3.3.2.2 Integrin Activation and Signaling**

As previously indicated, integrin receptors are able to signal bidirectionally, transmitting information across the plasma membrane in both directions (Coppolino and Dedhar, 2000; Giancotti and Ruoslahti, 1999). Integrin affinity for ligand is regulated by signals that initiate inside the cell and are conducted extracellularly (inside-out signalling), while integrin ligation transmits extracellular signals inside the cell (outside-in signalling). The regulation of growth can therefore be influenced not only by the surface expression level or repertoire of integrins, but also by the activation state of these receptors, which dictates functionality and can be altered during developmental maturation and in response to ECM, providing a sophisticated mechanism for the regulation of integrin activity at the cell surface (Coppolino and Dedhar, 2000; Lemons and Condic, 2006). As a result, integrins can exist in an inactivated low-affinity conformation, where the extracellular domain of each subunit exists in a folded conformation and the transmembrane and cytoplasmic domains are laterally associated, or in an activated high-affinity conformation, where the extracellular domains swing open distally and the transmembrane and cytoplasmic domains separate (Xiao et al., 2004). This allosteric switch can be induced via effector binding to the intracellular tails of the integrin (Tadokoro et al., 2003; Vinogradova et al., 2002), or by extracellular ligation of

the receptor (Takagi et al., 2002). In the activated conformation,  $\beta 1$  cytoplasmic tails become linked to the actin cytoskeleton via the actions of cytoplasmic adapter proteins (Critchley, 2004; Liddington and Ginsberg, 2002) and this can promote regenerative growth under poor environmental conditions where growth promoting molecules are sparse (Ivins et al., 2000; Lemons and Condic, 2006; Neugebauer and Reichardt, 1991).

#### **1.3.3.2.2.1 Inside-Out Signaling**

Integrins can bind and interpret ECM molecules, transmitting this information into the cell, but information can also be transmitted in the reverse direction, from the cytoplasmic tail to the extracellular domain (Kolanus and Seed, 1997; Schwartz et al., 1995) to regulate the activation state of integrins on the cell surface. Inside-out signalling occurs primarily via two mechanisms: intracellular signals can induce changes in ligand binding affinity through propagation of a conformational change from the cytoplasmic tail to the extracellular domain, thus creating a conformation more favourable to ligand binding (Hughes et al., 1996; O'Toole et al., 1994; O'Toole et al., 1991). Secondly, intracellular signals can alter the avidity of integrin-ligand interactions by redistributing receptors within the plasma membrane, creating clusters of integrins which can promote multivalent interactions necessary for full receptor activation (Brown, 1997; Shattil and Ginsberg, 1997). Changes in avidity are thought to be regulated at least in part by the cytoskeleton that permits lateral movement of the integrins (Harris et al., 2000; van Kooyk and Figdor, 2000), as well as by adapters and enzymes, including PI3K, PKC, Ras and Rap, among others (Shimizu, 1996; van Kooyk et al., 1999).

#### **1.3.3.2.2.Outside-In Signaling**

Interaction of an integrin heterodimer extracellular domain with ECM molecules can induce rapid activation of the integrin, which is critical for the inward transmission of signals for the modulation of cellular activities. Outside-in signalling can mediate cell adhesion, survival, growth and migration, by ligating ECM molecules and transducing signals through multiprotein complex interactions and the initiation of biochemical cascades allowing the cell to interpret the extracellular environment and respond appropriately (Coppolino and Dedhar, 2000; Lemons and Condic, 2008; Miranti and Brugge, 2002). Since the integrin cytoplasmic tail lacks catalytic activity, the transduction of extracellular signals required integrin aggregation within the plane of the membrane (Giancotti and Ruoslahti, 1999) and the subsequent recruitment of adapter proteins, non-receptor protein tyrosine kinases and cytoskeletal elements to form focal adhesions wherein signalling is facilitated by the high local concentration of these proteins (Bray et al., 1998). The activated state of integrins is necessary, but not sufficient to induce clustering. This also requires the presence of PIP<sub>2</sub> and talin, and immobilization of the ligand (Cluzel et al., 2005). Furthermore, both receptor clustering and ligand occupancy are central for full activation of integrin-mediated responses (Miyamoto et al., 1995).

#### **1.3.3.2.3 Integrin Associated Signalling Cascades**



Integrin-ECM interactions at the cell surface induce receptor clustering and recruitment of a variety of intracellular effectors, some of which link directly to the actin cytoskeleton to alter cell morphology, but others initiate tyrosine and serine phosphorylation events triggering multiple parallel downstream signalling cascades that interact and coordinate to change gene expression and alter cellular behaviour to drive a growth response (Coppolino and Dedhar, 2000; Giancotti and Ruoslahti, 1999; Kumar, 1998; Tucker et al., 2005; Tucker et al., 2006; Tucker et al., 2008). A large number of proteins have been demonstrated to associate transiently or stably to focal adhesions and interact via multiple binding domains on each molecule to create a web of proteins that is difficult to detangle in terms of the sequence of signal transduction events (reviewed in Miranti and Brugge, 2002) downstream of integrin ligation (Figure 1.5). Nonetheless, several critical interactions have been elucidated.

The cytoplasmic tail of the  $\beta$  subunit interacts directly with cytoskeletal components including talin and  $\alpha$ -actinin (Horwitz et al., 1986; Otey et al., 1990), which subsequently interact with other cytoskeletal elements, zyxin, paxillin, and vinculin, which in turn, bind tensin. This resulting focal adhesion scaffolding is critical for maintenance of cell-substrate adhesion (BurrIDGE et al., 1988).

The  $\beta 1$  cytoplasmic tail can also interact directly with the N-terminal domain of focal adhesion kinase (FAK), which plays a central role in integrin-stimulated signalling events (Chen et al., 1995b; Lewis and Schwartz, 1995; Miyamoto et al., 1995; Schaller et al., 1995). Upon integrin activation, FAK localizes to sites of focal adhesion and becomes autophosphorylated at tyrosine 397, creating a docking site for SH2-domain containing proteins, including Src and Src Family kinases (SFK) (Schaller et al., 1994;

**Figure 1.5: Schematic illustration of integrin-mediated intracellular interactions.**

Integrin ligation results in aggregation and the subsequent recruitment of various adapters and kinases, including FAK, Src and ILK, providing direct links to the actin cytoskeleton and initiating multiple biochemical cascades. Adapted from Miranti and Brugge, *Nature Cell Biology* 2002 4: E83-E90.



**Figure 1.5**



Schlaepfer et al., 1994). Src can further phosphorylate FAK at other tyrosine residues, promoting full catalytic activity of FAK, and creating additional SH2 binding sites for the transduction of signals to many downstream pathways. For example, binding of Shc or Grb2-SOS results in the activation of the Ras-MAPK cascade (Schlaepfer and Hunter, 1997), which results in the induction of transcription factor activity for the promotion of growth and differentiation (reviewed in Kumar, 1998), but can also promote migration in a transcription-independent manner (Klemke et al., 1997). The FAK-Src complex also results in the phosphorylation of FAK associated proteins, including paxillin, tensin, vinculin, and large adapter p130CAS (Schlaepfer and Hunter, 1997; Vuori et al., 1996), and promotes a direct interaction between the C-terminus of FAK and talin (Chen et al., 1995b). Furthermore, phosphorylation of FAK enables it to interact with PI3K, either directly or through Src, resulting in its activation (Chen et al., 1996a), and the downstream activation of Akt.

Another protein demonstrated to interact with the  $\beta 1$  intracellular domain is Integrin-linked Kinase (ILK), which can function as both an adapter and a serine/threonine kinase to mediate cellular attachment and signal transduction downstream of integrin activation (Hannigan et al., 1996). ILK influences the activity of GSK3 $\beta$  and Akt and thereby likely has a role in neuronal growth and survival (Delcommenne et al., 1998; Persad et al., 2000; Persad et al., 2001).

Integrins are complex bidirectional receptors that have direct links to the cytoskeleton and are capable of instigating diverse molecular interactions and initiating numerous downstream signalling pathways, and are therefore well-positioned to be central regulators of both developmental and regenerative growth responses.

#### **1.4 Rat Pheochromocytoma Cells as a Model for Axonal Regeneration**

Rat pheochromocytoma (PC12) cells were clonally derived in 1976 from a transplantable rat pheochromocytoma (Greene and Tischler, 1976), and have since been used extensively in the study of fundamental problems relating to neuronal cell differentiation and function. PC12 cells display multiple features of sympathicoblasts, the precursors to post-mitotic sympathetic neurons, and can be differentiated by prolonged exposure to NGF to acquire the phenotypic properties of mature sympathetic neurons. In this regard, the robust response to NGF occurs via the two distinct receptors, p75NTR and TrkA, and results in cessation of proliferation, extension of neurite outgrowth, generation of electrical excitability and functional synapse formation (Greene, 1982; Shafer and Atchison, 1991). Thus PC12 cells serve as a valuable model for the study of NGF responses, which is further complimented by the ease of genetic mutability. The introduction of new genes and modifications can be propagated as permanent lines. A prime example of this technique has been used in this series of studies. An NGF-nonresponsive PC12 cell line, which arose as a spontaneous mutation to lack TrkA expression, was transfected with a series of mutated Trk receptors. The resulting stable cell lines were previously used for the dissection of specific Trk-mediated signalling pathways (Obermeier et al., 1993a; Obermeier et al., 1993b; Stephens et al., 1994).

PC12 cells are also a useful model for the study of neuronal interactions with the ECM (Tomaselli et al., 1987). These cells possess the ability to extend neurites across collagen (Col), LN or fibronectin (FN) coated substrates, which closely approximates the



response of sympathetic neurons (Tomaselli et al., 1987). Both cell types share common structurally homologous ECM receptors which selectively interact with distinct structural domains of LN (Tomaselli et al., 1987). Investigations of neuronal interaction with ECM proteins are hindered by the requirement for pure neuronal cultures in sufficient quantities for biochemical analyses. The use of neuronal cell lines, like PC12, can circumvent this difficulty. PC12 cells are well characterized, homogeneous, stable and respond to NGF and ECM with reasonable fidelity to normal sympathetic neurons (Greene, 1982; Hosang and Shooter, 1985; Tomaselli et al., 1987).

Despite these many advantages, the use of PC12 cells also has limitations: they are derived from tumour cells and thus cannot be interpreted as an exact model of neuronal behaviour. They do not form dendrites and differ from neurons regarding formation of synaptic connections. For this reason, findings must be replicated and verified in primary neuron cultures. Furthermore, PC12 cells can be subject to spontaneous mutation and the inadvertent selection of a subpopulation with characteristics that differ from the norm. As changes over time are inevitable, all experiments in these studies have been performed with passage number lower than 10.

Primary neuronal cultures possess obvious advantages with respect to the physiological relevance of experimental outcomes. However, these cells must often be cultured at a particular developmental stage to ensure viability, are more difficult to maintain long-term, and cultures are rarely free of non-neuronal contaminants. Furthermore, primary neurons are notoriously difficult to transfect, and thus cannot be easily manipulated on a genetic level. The present series of studies utilizes cultures of various CNS neurons, including cerebellar granule neurons (CGN) and hippocampal



neurons to determine the effects of neurotrophic and ECM stimulation on cellular responses, both biochemical and functional. Neurons isolated from the postnatal cerebral cortex were not used for these studies, as they lose the ability to adhere to LN shortly after birth.

### **1.5 Purpose of the Study**

Like growth and guidance of the developing nervous system, axonal regeneration following nerve injury is largely dependent upon factors found in the extracellular environment. One of the key factors involved in regenerative neurite outgrowth, is the trophic support provided by the neurotrophins, including NGF, which stimulates the initiation and extension of neurites by binding to two structurally distinct receptors. TrkA ligation and subsequent phosphorylation of the cytoplasmic tail initiates discrete signalling cascades for the promotion of growth and survival. p75NTR, the low affinity receptor is associated with a variety of signalling interactions, both ligand-dependent and independent, and ligation outcome depends upon TrkA coexpression.

Another key factor involved in the promotion of axonal regeneration following nerve injury is the existence of a permissive environment, the signals from which are mediated by the integrin receptors. Integrins themselves are capable of initiating a wide variety of signalling interactions upon ligand binding and subsequent aggregation. Because ECM proteins and neurotrophins promote axonal regeneration using similar intracellular signalling cascades, optimized neurite outgrowth may potentially be achieved through synergistic or cooperative crosstalk between cascades activated by

neurotrophins and those activated by ECM proteins, which integrate to stimulate reorganization of the cytoskeleton and induce requisite gene expression for a regenerative response. These signalling interactions may occur at several levels, from receptor transactivation, to cooperative activation of downstream components, to modulation of co-receptor expression or activity, thus enabling a tightly controlled cellular response. The goal of this project was to investigate signal transduction steps activated by neurotrophin and ECM ligands to determine how these pathways may cooperate in order to promote optimal axonal regeneration. Elucidating neuronal growth or regeneration programs in PNS neurons is likely to provide insights into ways of enhancing CNS repair. I hypothesize that TrkA plays a role in cooperative signalling with integrins that results in enhanced neurite outgrowth, and that particular phosphorylation sites on the TrkA cytoplasmic tail are important to this cooperativity, by promoting enhanced activation of downstream signalling intermediates and receptors necessary for promotion of growth, through pathway crosstalk or intersection, or by removing inhibition. Additionally, any growth response is likely to depend on a contribution from p75NTR, which has the potential to impact growth both in the presence and absence of ligand. Integrin signalling may be enhancing NGF signalling by promoting further activation of Trk-induced intermediates or suppressing p75NTR-induced inhibition. These signalling interactions may alter actin dynamics which is important for both axonal regeneration and cell migration.

**Objective 1: Determine the effects of TrkA signalling on p75NTR expression using TrkA phosphorylation mutants.** The cellular responses to NGF are mediated by two



structurally unrelated receptors, TrkA and p75NTR, which have been shown to interact, resulting in reciprocal modulation of function. It is essential to investigate what effects p75NTR signalling is having with regard to both its well known role in moderating TrkA:NGF signalling and its own autonomous cascades, and I hypothesized that NGF may be acting via a Trk-induced signalling cascade to influence p75NTR expression. In this study, the modulation of p75NTR expression by specific TrkA autophosphorylation sites in the presence or absence of NGF was examined. We have used cell lines derived from PC12 cells that express either no endogenous TrkA (PC12nnr5) or TrkA receptors mutated via site-directed mutagenesis to abrogate the individual tyrosine residues on the cytoplasmic tail (Y490F, Y785F, Y490/785F) (Chapter 2). Upon determination that NGF results in the upregulation of p75NTR expression in those cell lines that express a functional Y785 phosphorylation site, pharmacological inhibition and siRNAs were utilized to investigate the mechanism by which NGF induces the upregulation of p75NTR expression (Chapter 3).

**Objective 2: Investigate the impact of permissive ECM component, LN, on the expression of p75NTR, and determine how this relates to neurite outgrowth.** LN is a key factor in promoting axonal regeneration, working co-ordinately to regulate growth in conjunction with neurotrophin signals. While LN-mediated mechanisms are not well-defined, they likely represent a complex interplay between enhancement of permissive cues and downregulation of those which are inhibitory. I hypothesized that LN may signal cooperatively with Trk-activated cascades to enhance regenerative growth, either by promoting the phosphorylation of signalling intermediates essential to growth, or by



decreasing the presence of inhibitory signals. This study used NGF-differentiated PC12 cells to investigate potential interactions between LN and NGF-mediated signalling pathways, and correlated these events with their impact on regenerative growth. I also examined the impact of LN on the expression of p75NTR, which has the potential to inhibit neurite outgrowth in the absence of neurotrophins (Chapter 4).

**Objective 3: Determine the role of LN-mediated downregulation of p75NTR in CNS neurons.** Having determined that LN-mediate a downregulation of p75NTR expression that is dependent upon PTEN induction and results in enhanced regeneration of PC12 cells, I subsequently investigated whether these signalling interactions occur in neonatal hippocampal neurons, and whether they correlate with enhanced growth (Chapter 4). Having determined that the LN-mediated alterations in PTEN and p75NTR were associated with influence over actin cytoskeletal remodelling via changes in active Rho, I hypothesized that this signalling cascade may also play a role in neuronal migration. I determined that LN-mediated changes in PTEN and p75NTR correlate temporally with the period of developmental migration of cerebellar granule neurons from the external granule layer to the internal granule layer. Investigation of LN-mediated cell migration was undertaken using a novel motility assay (Chapter 5), and I used a variety of pharmacological inhibitors, siRNA and genetic overexpression to determine if the LN-induced changes in PTEN and p75NTR were associated with the regulation of cerebellar granule neuron migration (Chapter 6).

**Objective 4: Determine the mechanism by which PTEN downregulates p75NTR expression.** My previous study indicated that the LN-mediated downregulation of p75NTR was at least partially transcriptional in nature. I therefore hypothesized that PTEN could be influencing the activity of transcription factors that regulate p75NTR expression. To study the molecular interactions between the induction of PTEN expression and the subsequent downregulation of p75NTR, I employed a series of PTEN activation mutants, localization immunocytochemistry and a variety of techniques to analyze the DNA-binding ability of transcription factor Sp1. These results were subsequently applied to the study of cerebellar granule neurons (Chapter 7).

**Objective 5: Investigate the regulation of PTEN expression by both TrkA and integrin associated signalling cascades.** Having determined that both TrkA and PTEN each independently regulate p75NTR expression, I hypothesized that TrkA could modulate PTEN expression upstream of changes to p75NTR. I therefore investigated whether TrkA phosphorylation sites had any influence over PTEN expression, and whether this influence could modulate cellular behaviours including growth and migration (Chapter 8). Furthermore, as integrin activation can induce PTEN expression, I hypothesized that this induction was due to downstream activation of integrin-associated signalling components. I examined the LN-mediated induction of PTEN, and investigated the role of integrin-associated signalling components in this induction using pharmacological and genetic inhibition (Chapter 9).

**Brief Summary of Results:** The results of these studies detail signalling events required for the regulation of p75NTR expression for the promotion of neurite outgrowth and motility. The neurotrophin-induced upregulation of p75NTR occurs via the PLC- $\gamma$ -PKC- $\delta$  signalling cascade initiated by ligation of the Trk receptor. The ECM signals via integrin activation and associated focal adhesion formation to induce Egr-1 activity which results in a rapid and potent upregulation of phosphatase PTEN. PTEN relocates to the nucleus where it exerts protein phosphatase activity over transcription factor Sp1 to decrease its DNA binding activity to the p75NTR promoter, resulting in decreased p75NTR transcription. This is followed by a decrease in p75NTR protein expression which is associated with a decrease in constitutively active Rho, and a subsequent growth or motility response. Collectively these studies demonstrate the importance of different modes of modulating p75NTR expression to control its signalling interactions, which can influence the cytoskeleton for both growth and motility responses in a variety of neuronal cell types.

Schematic overview of thesis results and associated chapters is provided in Figure 1.6.



**Figure 1.6: Schematic overview of thesis results and associated chapters.**

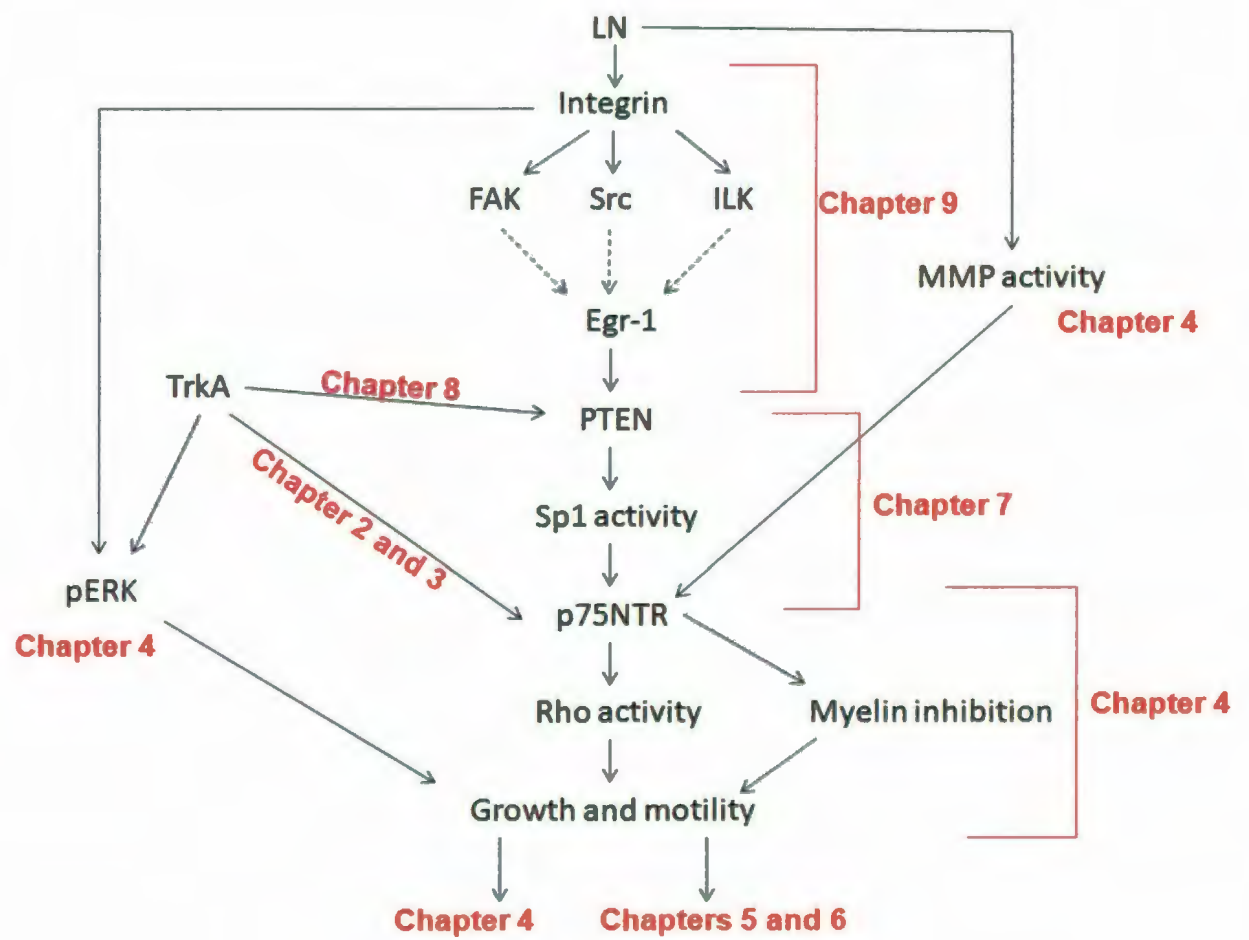


Figure 1.6

## THESIS CO-AUTHORSHIP STATEMENT

This thesis is comprised of 11 chapters. Chapter 1 introduces the background and rationale for the thesis work. Chapters 2-9 contain the original data compiled from each of the projects completed for the thesis. Chapter 10 gives a discussion which highlights the cohesiveness of the projects completed during this study, while Chapter 11 summarizes the major conclusions of this work.

A great majority of the work described in this thesis was performed by the author, Sherri Rankin, whose primary role was a contribution to the design of the research proposal, development of research methodology, data collection and analysis, and preparation of the manuscripts. Chapter 2 was published as a first-author paper entitled: **“TrkA NGF receptor plays a role in the modulation of p75NTR expression”** in *Neuroscience Letters*, 2005, volume 383, pp.305-310. The work comprising Chapter 3 has been accepted for publication as a first-author paper entitled: **“Neurotrophin-induced upregulation of p75NTR via a Protein Kinase C-delta dependent mechanism”** in *Brain Research*, 2008, volume 1217, pp. 10-24. A portion of the work comprising Chapter 4 has been accepted for publication as a first author paper entitled **“Neurite Outgrowth is Enhanced by Laminin-Mediated Downregulation of the Low Affinity Neurotrophin Receptor, p75NTR”** in *Journal of Neurochemistry*, 2008. The work comprising Chapter 5 was published as a first-author manuscript entitled **“A method to assess multiple aspects of the motile behaviour of adherent PC12 cells on applied biological substrates”** in *Journal of Neuroscience Methods*, 2006, volume 156, pp. 55-63. The work comprising Chapter 7 has been accepted for publication as a first author paper entitled **“PTEN downregulates p75NTR expression by decreasing the**



**DNA binding activity of Sp1“ in *Biochemical and Biophysical Research***

*Communications*, 2008. The author appreciates and recognizes those contributions made by others as follows: The real-time RT-PCR studies used throughout this thesis were all performed by Cliff Guy, who also provided assistance with DNA cloning for construct creation and lentiviral production, and assisted with the EMSA and CHIP analyses presented in Chapter 7. Masuma Rahimtula provided extensive assistance with animal dissection for Chapters 3 and 4, technical assistance with assay design in Chapter 5, and was responsible for the preparation of chemical compounds used throughout the thesis. Dr. Mearow played a pivotal role in the experimental design, and participated in the trouble-shooting, and correction and improvement of the manuscripts.

## **Chapter 2: TrkA NGF receptor plays a role in the modulation of p75NTR expression**

*This study has been published in Neuroscience Letters in August 2005, volume 383, pp. 305-310.*

### **2.0 Summary**

The cellular response to nerve growth factor (NGF) is mediated by two structurally unrelated receptors, TrkA and p75 neurotrophin receptor (p75NTR), which have been shown to interact resulting in reciprocal modulation of function. In this study, we have examined the modulation of p75NTR protein expression by specific TrkA autophosphorylation sites in the presence or absence of NGF. We have used cell lines derived from PC12 cells that express either no endogenous TrkA (PC12nnr5) or TrkA receptors mutated via site-directed mutagenesis to abrogate individual tyrosine autophosphorylation sites on the cytoplasmic tail (Y490F, Y785F, Y490/785F). Results indicate that in the absence of TrkA in PC12nnr5 cells there is reduced constitutive p75NTR expression, which can be restored to different degrees by transfection of the Y490F TrkA or the Y490/785F TrkA, but not by transfection of the Y785F TrkA. In addition, the expression of p75NTR was upregulated in the presence of NGF in the parental and Y490F cell lines only. Together these results indicate a role for the individual tyrosine autophosphorylation sites of TrkA in regulating p75NTR expression.

## 2.1 Introduction

Nerve Growth Factor (NGF) is a neurotrophic factor that is essential for the growth, survival and maintenance of the developing nervous system, influencing specific populations of sensory, sympathetic and central nervous system neurons (Barde, 1989; Cattaneo and McKay, 1990; Huang and Reichardt, 2001; Levi-Montalcini, 1987; Ruit et al., 1990). In PC12 cells, it is instrumental in the cessation of cellular proliferation and neurite outgrowth that is characteristic of neuronal differentiation (Greene and Tischler, 1976; Greene, 1982).

The biological effects of NGF are mediated by two structurally unrelated receptors, TrkA and p75 neurotrophin receptor (p75NTR). p75NTR, classically known as the low-affinity NGF receptor, is a member of the tumor necrosis factor (TNF) receptor superfamily, traditionally associated with apoptosis. While p75NTR is able to bind all neurotrophins in the NGF family with equal affinity (Levi-Montalcini, 1987), ligand binding is not associated with intrinsic enzymatic activity (Roux and Barker, 2002). In contrast, NGF binding to the receptor tyrosine kinase TrkA results in receptor dimerization and transautophosphorylation of specific tyrosine residues on the cytoplasmic tails. These phosphorylated residues then serve as docking sites for proteins, which in turn become tyrosine phosphorylated to initiate signalling cascades (Kaplan and Miller, 2000).

TrkA contains only 2 cytoplasmic tyrosine residues outside of the kinase domain: Y490 binds Shc, resulting in activation of the Ras/MAPK signalling cascade, while Y785 serves as a site for PLC $\gamma$  interactions. These specific pathways have been dissected using



mutated PC12 derivatives, and the individual cascades were found to compensate for each other in regards to neurite outgrowth (Loeb et al., 1994; Obermeier et al., 1993a; Stephens et al., 1994).

PC12 cells express both TrkA and p75NTR NGF receptors, and are commonly used for mutational analysis of the role of TrkA in NGF-mediated survival and differentiation responses due to their ease of mutability. When studying NGF responses, it is essential to be aware of the full complement of NGF surface receptors mediating all NGF signals, as well as potential mutations that may influence the expression of one or the other NGF receptor. p75NTR is typically studied in regards to its role in collaborating functionally with TrkA, but no studies to date have addressed the potential role of TrkA in modulating p75NTR expression.

Since TrkA overexpression was noted to coincide with an increase in the number of low-affinity binding sites for NGF (Hempstead et al., 1992), and the absence of TrkA in PC12nnr5 cells was accompanied by a decreased expression of p75NTR mRNA (Loeb et al., 1991), we employed a well characterized PC12 derived mutant model to investigate the importance of the individual TrkA cytoplasmic tail autophosphorylation sites in the modulation of p75NTR expression.

## **2.2 Materials and Methods**

### **2.2.1 Cell culture**

The experimental model consisted of parental PC12 cells, and 4 mutated PC12 derivative cell lines (gifts from Dr. David Kaplan), which were employed in earlier

studies examining the role of individual TrkA autophosphorylation sites in NGF signalling cascades (Green et al., 1986; Loeb and Greene, 1993; Loeb et al., 1994; Stephens et al., 1994). PC12nnr5 cells were derived by mutating parental PC12 cells using ethyl methanesulfonate (EMS), as described by Green et al. (Green et al., 1986). PC12 derivatives expressing mutated TrkA receptors (Y490F, Y785F, Y490/785F) were created by Stephens et al. (Stephens et al., 1994). Briefly, PC12nnr5 cells were transfected to express a TrkA receptor that had been altered via site-directed mutagenesis to abrogate either one or both of the autophosphorylation sites of the cytoplasmic tail.

Cells were maintained on rat-tail collagen-coated tissue culture flasks in RPMI medium supplemented with 10% horse serum, 5% fetal calf serum and 1% penicillin/streptomycin/glutamine solution. In the case of mutated lines Y490F, Y785F and Y490/785F, media was additionally supplemented with Geneticin G418 (4 $\mu$ L/mL) to ensure continued selection of the mutant population. All cells were incubated at 37° C in 5% CO<sub>2</sub>.

### **2.2.2 Western blot analyses**

For Western blot analysis, cells were trypsinized and subcultured on Poly-D-Lysine coated 12-well plates in serum-starved conditions for 24 hours in the presence or absence of NGF (50ng/mL). Cells were then harvested in the presence of sodium orthovanadate and subsequently subjected to lysis overnight at 4° C prior to centrifugation (10,000 rpm, 5min). A BCA protein assay (Pierce, Rockford IL) was used to determine protein concentration and equivalent amounts of protein (50 $\mu$ g) were loaded onto 8% SDS-polyacrylamide gels. Gels were subsequently transferred to nitrocellulose

membranes that were then exposed to Ponceau red to ensure equal protein loading. After washing in TBS, blots were blocked in 3% non-fat dry milk for 1 hour at room temperature, and then incubated with a primary antibody directed toward either p75NTR (MC192; Oncogene, San Diego CA) or TrkA (06-574; Upstate Biotechnology, Charlottesville, VA) overnight at 4° C. A second p75NTR antibody (REX; a kind gift from Dr. L Reichardt) was also used in some experiments to detect p75NTR expression. A final incubation with HRP-conjugated secondary antibody (AP307P, AP308P; Chemicon, Temecula, CA) for 1 hour at room temperature was followed by visualization using an ECL method. Each experiment was repeated three times, subjected to densitometry and normalized to mitogen-activated protein kinase (MAPK) to ensure equal protein for comparison. Statistical analysis was performed using GraphPad Prism 4 with significance being determined using one-way ANOVA testing.

### **2.2.3 Real-time RT-PCR**

For real time RT-PCR analysis, cells were subcultured on Poly-D-Lysine coated 12-well plates in serum-starved conditions for 24 hours in the presence or absence of NGF (50ng/mL). RNA was isolated using Trizol reagent (Invitrogen) as per the manufacturer's instructions and was subsequently treated with DNase (Ambion) to remove traces of contaminating DNA. RNA was reverse transcribed to cDNA using MMLV reverse transcriptase (Invitrogen) prior to use as template for real-time PCR amplification using the following PCR primer pairs: p75plus 5'-TGCATCTGAGCTGGTGTCTGTCTT, p75minus 5'-TGCGTACAATGCTCC



TGGTCTCTT, GAPDH plus 5'-CCATCACCATCTTCCAGGAG, GAPDH minus 5'-CCTGCTTCACCACCTTCTTG. PCR amplification was performed using the Roche LightCycler and quantified using SYBR green I. P75NTR mRNA expression was subsequently normalized using the housekeeping gene, GAPDH.

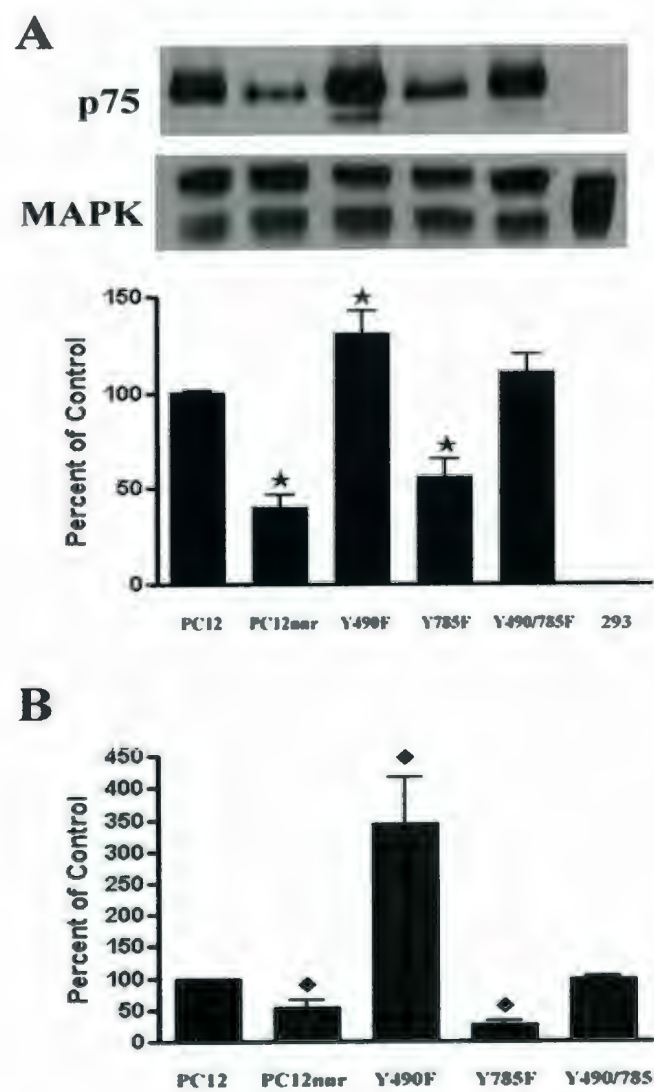
#### **2.2.4 Immunocytochemistry**

For immunocytochemical studies, cells were trypsinized and subcultured serum-free on Poly-D-Lysine coated 16-well chamber slides for 24 hours in the presence or absence of NGF (50ng/mL), prior to fixation in 4% formaldehyde for 15 min. Cells were then permeabilized with 0.1% Triton-X-100 and blocked with 10% normal goat serum in PBS for 1 hour at room temperature. Cells were incubated with a monoclonal antibody directed against p75NTR (MC192, Oncogene) and a polyclonal antibody directed against TrkA (Upstate Biotechnology) for 16 hours at 4° C, followed by incubation with Cy2- and Cy5-tagged secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) for 1 hour at room temperature. Finally slides were coverslipped with glycerol, and the receptor expression patterns were visualized using confocal laser scanning microscopy.

#### **2.3 Results**

Western blot analyses illustrate that the constitutive level of p75NTR protein expression is reduced in PC12nnr5 cells to 38.8 $\pm$ 5.22% relative to the parental cell line (Fig 2.1A). This result is consistent with other studies that have noted reduced p75NTR mRNA (Loeb et al., 1991) and a reduced number of low affinity binding sites (Green et al., 1986) associated with the PC12nnr5 cell line. In PC12nnr5 cells transfected with the

**Fig. 2.1. Constitutive p75NTR expression.** A. Constitutive p75NTR protein expression is significantly reduced in PC12nnr5 cells that lack NGF receptor TrkA. Expression of p75NTR can be restored to different degrees by expression of a TrkA receptor mutated to abrogate autophosphorylation at Y490 or Y490/785, but not by expression of a TrkA receptor mutated to abrogate autophosphorylation site at Y785. Values expressed represent the mean p75NTR protein expression, relative to MAPK, of 9 experiments +/- SEM. \* $p < 0.05$  as determined by one-way ANOVA. HEK293 cells which do not express p75NTR, served as a negative control. B. Constitutive p75NTR mRNA expression, as determined by real time RT-PCR quantification of SYBR green I, displays a similar trend. Values expressed represent the mean p75NTR mRNA expression of 9 experiments +/- SEM. \* $p < 0.005$  as determined by one-way ANOVA.



**Figure 2.1**



Y490F TrkA receptor, p75NTR protein expression is restored to levels greater than the parental line ( $122.0 \pm 9.2\%$ ). In contrast, in PC12nnr5 cells transfected with the Y785F TrkA receptor, p75NTR expression is not significantly different from the PC12nnr5 constitutive expression, remaining  $51.9 \pm 6.26\%$  lower than wild-type. Transfection of the PC12nnr5 cell line with the Y490/785F TrkA receptor restored p75NTR protein expression to levels not significantly different from wild-type ( $119.9 \pm 10.5\%$ ) (Fig 2.1A). These results are confirmed by the p75NTR expression patterns noted in both the mRNA expression profile (Fig. 2.1B), and the immunocytochemical assessment (Fig 2.2).

It is conceivable that the amount of TrkA introduced into the cells could be influencing the expression of p75NTR separately from the phosphorylation site alterations. The mutant cell lines employed in this study were selected as low-expressors in terms of TrkA, (personal communication, Dr. D. Kaplan), and this was confirmed both by Western blot analysis of TrkA levels expressed by each cell line (Fig. 2.3), and by immunocytochemistry (data not shown). All mutant lines express between 0% and  $20.5 \pm 2.8\%$  of the TrkA levels associated with wild type PC12 cells, thus eliminating the possibility that a TrkA overexpression is the cause of the p75NTR overexpression noted in Y490F cells.

Since NGF is a known trigger for p75NTR upregulation in neurons *in vitro* (Lindsay et al., 1990), *in vivo* (Kitzman et al., 1998; Wyatt and Davies, 1993), and in PC12 cells (Doherty et al., 1988), we investigated whether or not the addition of NGF to the culture media would result in upregulation of p75NTR in the presence of a mutated TrkA receptor. By Western blot analysis, we determined that only the wild-type and Y490F cell lines responded to 24 hours of NGF exposure with a significant increase of

**Fig 2.2. p75NTR immunoreactivity.** Photomicrographs illustrating the p75NTR expression patterns of parental PC12 cells (A,B), PC12nnr5 cells lacking TrkA (C,D) and cell lines expressing mutated TrkA receptors: Y490F (E,F), Y785F (G,H) and Y490/785F (I,J). A, C, E, G, and I represent control conditions, and B, D, F, H, and J are representative of cells plated in the presence of NGF (50 ng/mL) for 24 hours. PC12nnr5 and Y785F cell lines express low levels of p75NTR, and receptor upregulation is not noted in response to NGF. In contrast, PC12, Y490F and Y490/785F cell lines constitutively express higher levels of p75NTR, though only PC12 and Y490F exhibit NGF-dependent p75NTR upregulation. Scale bar, 20 $\mu$ m.

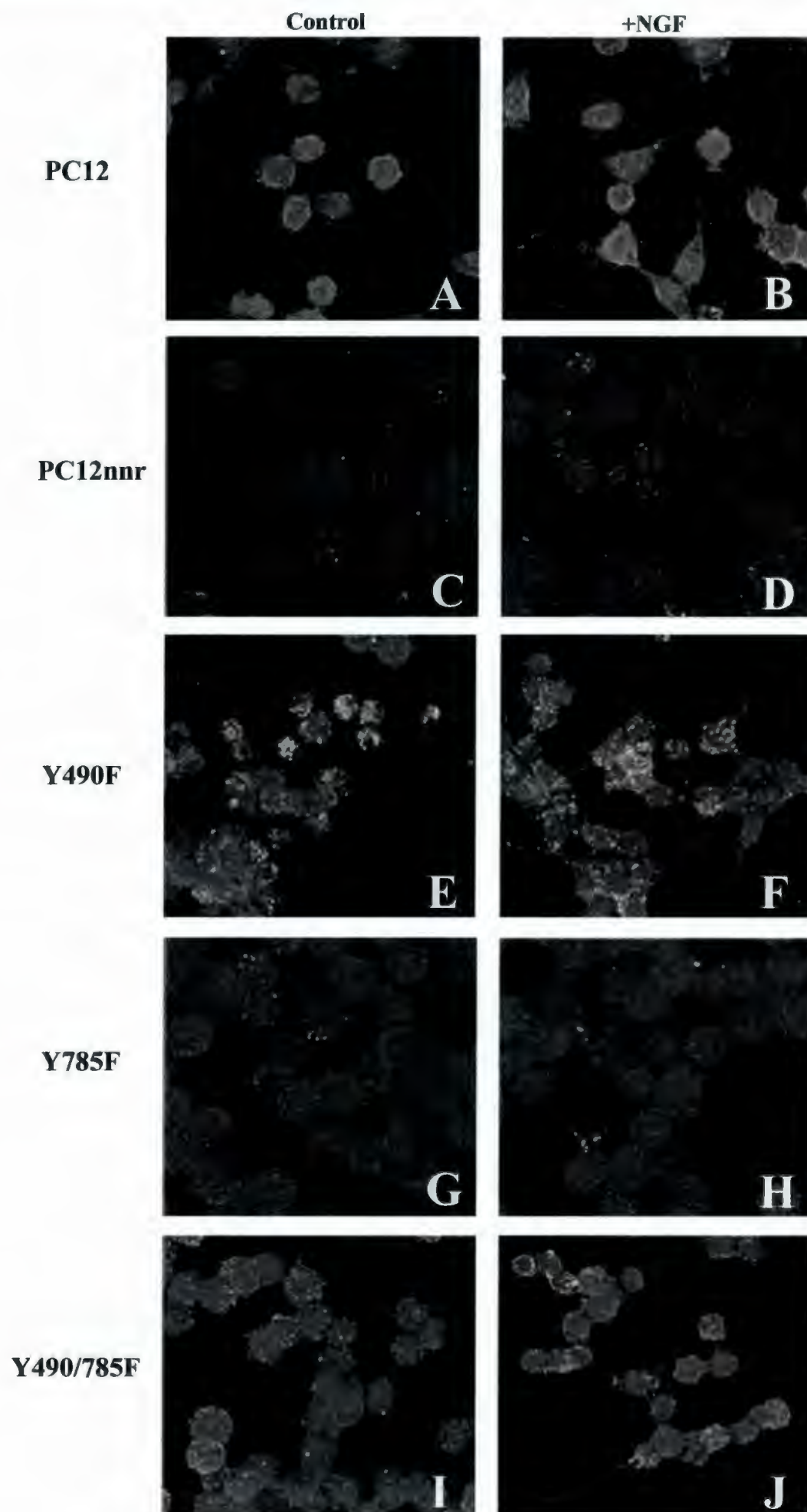
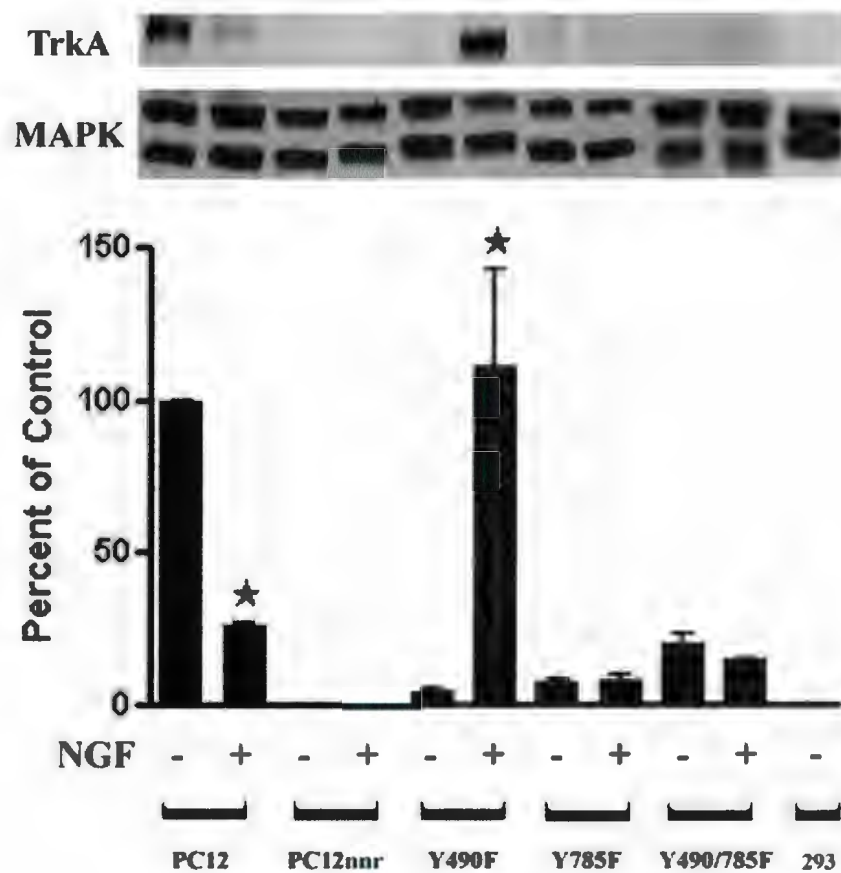


Figure 2.2



**Fig 2.3. TrkA expression in the presence or absence of NGF.** All PC12 derivative cell lines expressing mutated TrkA receptors used in this study express low constitutive levels of TrkA relative to the parental PC12 cells. In response to NGF (50ng/mL), PC12 cells exhibit a significant decrease in TrkA receptor expression after 24 hours. In contrast, cells expressing the Y490F mutation exhibit a significant increase in TrkA expression under the same NGF stimulation conditions. None of the other cell lines tested demonstrated any significant changes in TrkA expression in response to NGF treatment. HEK293 cells which do not express TrkA were used as a negative control. Values expressed represent the mean TrkA protein expression, relative to MAPK, of 3 experiments +/- SEM. \* $p < 0.001$  as determined by one-way ANOVA.



**Figure 2.3**

p75NTR protein expression (Fig 2.4). Wild-type PC12 cells increased p75NTR expression  $119.4 \pm 21.5\%$  above unstimulated condition, while Y490F cells also displayed significantly elevated p75NTR expression above the unstimulated condition. The PC12nnr5 cells, and cells expressing the Y785F or Y490/785F TrkA receptors showed no significant changes in p75NTR expression in response to NGF after 24 hours of exposure.

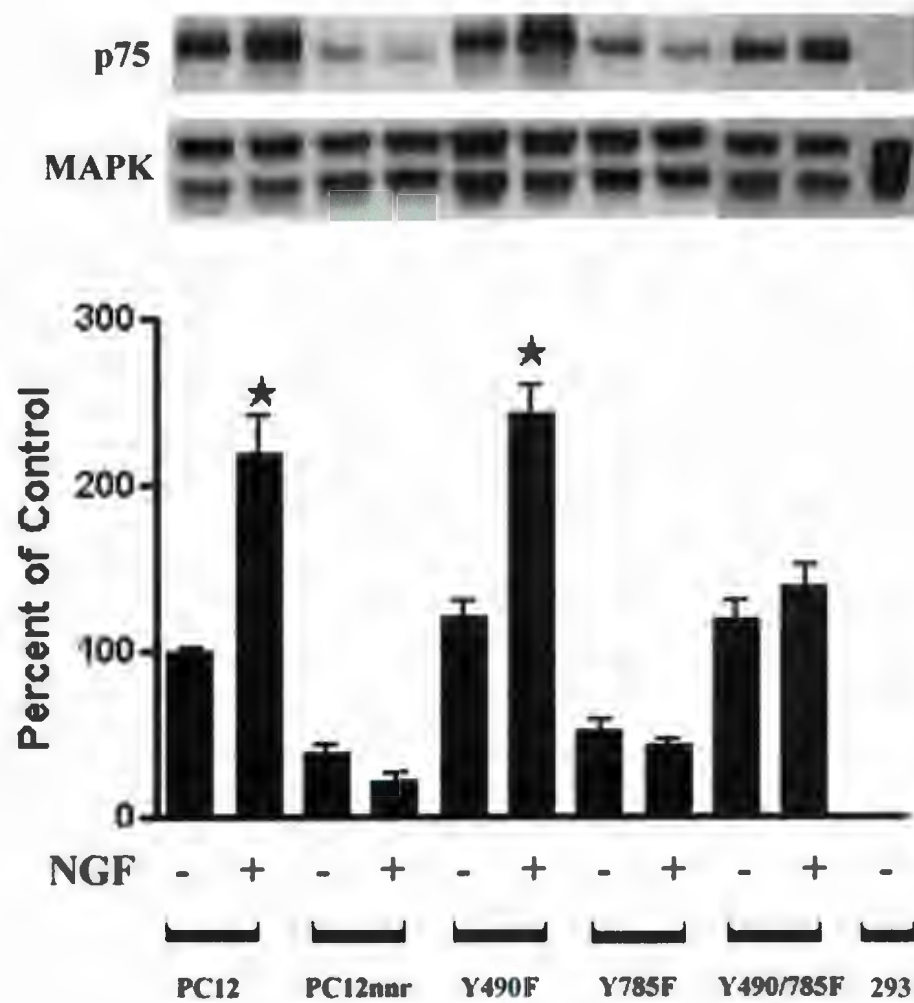
Further Western blot analyses of TrkA expression in response to NGF revealed differential responses between the cell lines as well. Wild-type PC12 cells decreased TrkA expression to  $26.1 \pm 1.0\%$  of control following 24 hours of exposure to NGF. In contrast, Y490F cells exhibited a significant increase in TrkA expression relative to the unstimulated condition. Again, PC12nnr5, Y785F and Y490/785F cells showed no significant changes in TrkA expression in response to NGF at 24 hours (Fig 2.3). Previous studies have established that NGF causes a lasting downregulation of TrkA (Grimes et al., 1996). As such, we cannot rule out a differential time course of internalization or receptor turnover as a possible explanation for the differential TrkA expression of wild-type and Y490F cells in response to NGF treatment.

## **2.4 Discussion**

In this study, we have used PC12nnr5 cells transfected with mutated TrkA receptors and cultured the stable derivatives in the presence or absence of NGF to illustrate that the presence of particular autophosphorylation sites on the cytoplasmic tail of the TrkA NGF receptor can modulate the protein expression of p75NTR. PC12nnr5



**Fig 2.4. p75NTR expression in the presence or absence of NGF.** Following exposure to NGF (50ng/mL) for 24 hours, p75NTR protein expression was significantly upregulated in parental PC12 cells and derivatives expressing only the Y490F TrkA mutation. The NGF-nonresponsive cell line (PC12nnr5), and the derivatives expressing the Y785F TrkA mutation or both the Y490/785F TrkA mutations showed no significant changes in p75NTR protein expression in response to NGF. HEK293 cells were used as a negative control. Values expressed represent the mean p75NTR protein expression, relative to MAPK, of 3 experiments +/- SEM. \* $p < 0.001$  as determined by one-way ANOVA.



**Figure 2.4**

cells were originally isolated and characterized to be phenotypically identical to the parental cell line in the absence of NGF, but were found to possess only 12 to 49% of NGF surface binding sites (Green et al., 1986). In the presence of NGF, these cells are deficient in c-fos activity, which is known to be associated with p75NTR activation by NGF (Barker and Shooter, 1994), and ultimately, lack NGF responses due to an absence of the TrkA receptor (Green et al., 1986). Transfection of PC12nnr5 cells with TrkA cDNA was found to completely restore all NGF responses back to wild-type levels (Loeb and Greene, 1993; Loeb et al., 1991).

The mutated cell lines used in the study have been well characterized with respect to neurite outgrowth and TrkA activation of signalling cascades, but have never been used to address modulation of p75NTR expression. We have used this model to illustrate that the restoration of the TrkA receptor in a variety of mutated forms has a significant influence on p75NTR protein expression. The mutant cell lines, Y490F, Y785F and Y490/785F all express low levels of their respective TrkA NGF receptors, removing the possibility that quantity of TrkA expressed regulates the expression of p75NTR independently of phosphorylation site associations. While PC12nnr5 cells display low constitutive levels of p75NTR relative to the parental cell line, the expression of a mutated TrkA receptor lacking both of the Y490 and Y785 phosphorylation sites resulted in a restoration of p75NTR protein back to wild-type levels. However, the expression of TrkA with an abrogated Y490 phosphorylation site resulted in an overexpression of p75NTR protein relative to wild-type levels. In contrast, the expression of TrkA lacking the Y785 phosphorylation site did not restore the expression of p75NTR protein above those levels seen in PC12nnr5 cells. Expression of p75NTR thus appears to be regulated



by PLC $\gamma$  since loss of the Y785 site, required for recruitment of PLC $\gamma$  to the Trk receptor, results in low levels of p75NTR expression. The results further suggest that Ras activation via phosphorylation of the Y490 site, necessary for Shc binding to Trk and downstream activation of Ras, may be negatively regulating p75NTR expression, since p75NTR expression was found to be highest in the cells expressing TrkA with the mutated Y490 site. The data also point out that the effects of NGF on both p75NTR and TrkA expression are mediated via TrkA, since NGF does not alter receptor expression in the PC12nnr5 cells; treatment of the cells with K252a also blocks the effects of NGF (data not shown).

TrkA and p75NTR are co-expressed throughout much of the developing nervous system, though typically neurons possess a 10-fold higher number of p75NTR than TrkA (Chao and Hempstead, 1995). Single receptor systems exist, and p75NTR can signal autonomously to exert a variety of effects (reviewed in Teng and Hempstead, 2004), but co-expression allows p75NTR and TrkA to associate, creating high affinity binding sites for NGF, which are important to the rapid internalization and subsequent retrograde transport of NGF signals for growth and survival (Ross et al., 1998; Teng and Hempstead, 2004).

p75NTR has been postulated to assume many roles, including sequestration of NGF, altering the affinity of Trk receptors for the appropriate ligand (reviewed in Hasegawa et al., 2004), and rapid apoptotic elimination of neurons unsuccessful in obtaining appropriate neurotrophic support (Bamji et al., 1998). Interestingly, p75NTR has also recently been found to increase the phosphorylation of Shc, an adaptor protein that is known to associate with the Y490 phosphorylation site of the TrkA cytoplasmic

tail (Epa et al., 2004). Most of these roles identify areas where p75NTR functionally supports TrkA, or modulates TrkA function. Several studies agree that cross-talk between p75NTR and TrkA is likely bi-directional, but few studies examine the reciprocal interactions. It has been noted, however, that TrkA can exert an inhibitory influence on p75NTR signalling, delaying activation of NF-kappa-B (reviewed in Mamidipudi and Wooten, 2002).

p75NTR upregulation has been associated with cases of nerve injury (Goettl et al., 2004), and exposure to NGF. NGF is known to result in the upregulation of p75NTR in the developing nervous system in the period preceding target innervation, marking the transition to NGF dependence for survival (Wyatt and Davies, 1993). PC12 cells have also been shown to increase p75NTR in response to NGF treatment (Doherty et al., 1988), a finding that is consistent with the wild-type results of the present study. The fact that the presence of NGF only causes elevated p75NTR expression in the two lines that express a functional Y785 phosphorylation site implicates PLC $\gamma$  or downstream intermediates of the PLC $\gamma$  signalling cascade as key components in the regulatory actions of TrkA on p75NTR expression. The molecular dissection of the role of TrkA in regulating p75NTR expression in PC12 cells is the subject of an ongoing investigation.

### **Chapter 3: Neurotrophin-Induced Upregulation of p75NTR via a Protein Kinase C-delta-Dependent Mechanism**

*This study has been published in Brain Research in 2008, volume 1217, pp. 10-24*

#### **3.0 Summary**

Neurotrophins exert their biological effects via p75NTR and Trk receptors. Functional interplay between these two receptors has been widely explored with respect to p75NTR enhancing the activation and signalling of Trk, but few studies address the bidirectional aspects. We have previously demonstrated that the expression of p75NTR can be differentially modulated by different Trk receptor mutations. Here we investigate the mechanism of Nerve Growth Factor (NGF)-induced upregulation of p75NTR expression. We utilize pharmacological inhibition to investigate the role of various TrkA-associated signalling intermediates in this regulatory cascade. Notably, the inhibition of phospholipase C- $\gamma$  (PLC- $\gamma$ ) using U73122, prevented the NGF-induced upregulation of p75NTR protein and mRNA. The inhibition of protein kinase C- $\delta$  (PKC- $\delta$ ) activation by rottlerin, a selective PKC- $\delta$  inhibitor, and by small interfering RNA (siRNA) directed against PKC- $\delta$  also inhibited this NGF-induced upregulation. Finally, we also show that in cerebellar granule neurons, BDNF acting via TrkB increases p75NTR expression in a PKC- $\delta$  dependent manner. These results indicate the importance of Trk-dependent PLC- $\gamma$  and PKC- $\delta$  activation for downstream regulation of p75NTR protein expression in response to neurotrophin stimulation, a process that has implications to the survival and growth of the developing nervous system.



### 3.1 Introduction

Nerve Growth Factor (NGF) is the prototypic member of a highly conserved family of neurotrophins that also includes Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin 4/5 (NT-4/5) among others (Ip et al., 1993). Neurotrophins play crucial roles in the development and maintenance of the vertebrate nervous system and are essential for cellular differentiation and survival (Cowley et al., 1994; Ernfors et al., 1994; Klein et al., 1994; Klein et al., 1993; Smeyne et al., 1994). Neurotrophin effects are mediated by two distinct receptors: p75NTR, a pan-neurotrophin receptor, and a specific member of the receptor tyrosine kinase Trks (reviewed in Hasegawa et al., 2004). TrkA binds NGF, TrkB binds BDNF and NT-4/5, and TrkC binds NT-3. While these receptors can often display binding promiscuity, these interactions are relatively specific in the presence of p75NTR co-expression (Barker, 1998; Benedetti et al., 1993; Mischel et al., 2001; Rodriguez-Tebar et al., 1992). Trk activation by ligand binding consists of receptor dimerization and transphosphorylation of specific tyrosine residues of the cytoplasmic tail, which initiate signalling cascades by serving as docking or interaction sites for other signalling intermediates with Src homology 2 (SH2) binding domains (Loeb et al., 1994; Obermeier et al., 1993a; Obermeier et al., 1993b; Stephens et al., 1994). In the case of NGF receptor TrkA, the only tyrosine residues outside of the kinase domain to become phosphorylated upon receptor activation are at positions 490 and 785 (Stephens et al., 1994). Y490 phosphorylation creates a docking site for the Shc adapter protein, which in turn activates the Ras-MAPK pathway. This site also binds adapters FRS2 and FRS3 (Dixon et al., 2006; Meakin et al., 1999). Y785 phosphorylation allows for the direct interaction of

TrkA with Phospholipase C- $\gamma$  (PLC- $\gamma$ ), whose downstream effectors include protein kinase C (PKC) isoforms and mitogen-activated protein kinase (MAPK) (Loeb et al., 1994; Obermeier et al., 1993a; Obermeier et al., 1993b; Stephens et al., 1994). Also associated with TrkA autophosphorylation is the recruitment of the adaptor protein GAB1 which leads to activation of the Phosphoinositide-3-Kinase (PI3K)/Akt signalling cascade for neuronal survival. Together these signalling cascades modulate the NGF-induced growth, survival and maintenance of various populations of sensory, sympathetic and central nervous system neurons (reviewed in Roux and Barker, 2002).

Trks are frequently co-expressed with p75NTR throughout the nervous system (reviewed in (Dechant and Barde, 2002). p75NTR, a 75kDa glycoprotein, is a member of the tumor necrosis factor (TNF) superfamily typically known for their roles in apoptosis (Dechant and Barde, 2002; Higuchi et al., 2003b) and is often studied for its apparent paradoxical roles in survival and death. p75NTR binds all neurotrophins with equal affinity, and although it is not associated with intrinsic enzymatic activity, it recruits a multiplicity of intracellular signalling partners and co-receptors to exert a diverse array of effects depending on cellular context (reviewed in Hasegawa et al., 2004; Nykjaer et al., 2005). Besides autonomous activation of apoptotic signalling cascades (Carter et al., 1996; Casaccia-Bonofil et al., 1996), p75NTR is known to constitutively activate RhoA leading to a suppression of neurite outgrowth that is reversible upon p75NTR binding NGF (Yamashita et al., 1999). Furthermore, p75NTR has been shown to play a role in the enhancement of TrkA activation and signalling (Epa et al., 2004; Nykjaer et al., 2005), particularly at low ligand concentrations, and complexes with TrkA to form the high affinity binding site (Teng and Hempstead, 2004).



The role of p75NTR in enhancing TrkA function is well known, but the bidirectionality of this system is largely ignored. TrkA overexpression results in an increase in p75NTR protein expression (Hempstead et al., 1992), while PC12 mutants lacking TrkA display decreased p75NTR mRNA (Loeb et al., 1991). Our previous study showed that mutation of the phosphorylation sites of the TrkA receptor resulted in differential expression of p75NTR (Rankin et al., 2005). Here we investigate the mechanism by which Trk influences p75NTR expression via downstream activation of signalling intermediates. Our results show that Trk-dependent activation of PLC $\gamma$  acts via PKC- $\delta$  to result in increased p75NTR expression. Both NGF (via TrkA) and BDNF (via TrkB) exert similar effects and show a general mechanism by which p75NTR expression can be regulated. This regulation may play a role in determining survival and axonal growth in response to target-derived neurotrophins in the developing nervous system.

### **3.2 Materials and Methods**

#### **3.2.1 Cell Culture**

The experimental model consisted of wild-type rat pheochromocytoma (PC12) cells, and 4 mutated PC12 derivative cell lines (gifts from Dr. David Kaplan, Hospital for Sick Children, Toronto, ON). PC12nnr5 cells were derived by mutating parental PC12 cells using ethyl methanesulfonate (EMS), as described by Green et al. (Green et al., 1986). PC12 derivatives expressing mutated TrkA receptors (Y490F, Y785F, and Y490/785F) were created by Stephens et al. (Stephens et al., 1994). Briefly, PC12nnr5 cells, which



lack endogenous TrkA, were transfected to express a TrkA receptor that had been altered via site-directed mutagenesis to abrogate either one or both of the autophosphorylation sites of the cytoplasmic tail.

Cell lines were maintained on rat-tail collagen-coated tissue-culture flasks in RPMI 1640 medium (Invitrogen, Burlington ON) supplemented with 10% horse serum (Invitrogen), 5% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin/glutamine solution (Invitrogen). In the case of mutated lines Y490F, Y785F and Y490/785F, media was additionally supplemented with Geneticin G418 (4  $\mu$ l/ml) to ensure continued selection of the mutant population. Cells were incubated at 37°C in 5% CO<sub>2</sub>, and cultured to 80% confluence prior to trypsinization for subculturing purposes.

### **3.2.2 Primary neuronal culture**

Cultures enriched in granule neurons were obtained from cerebella of 8-day old Sprague Dawley rat pups as described previously (Jiang et al., 2003). Cerebella were stripped of meninges, finely chopped and dissociated by enzymatic digestion using 0.25% Trypsin-EDTA followed by mechanical trituration. The culture medium was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS, 25 mM KCl and 1% penicillin/streptomycin solution (Invitrogen). Cells were seeded onto PL-coated 12-well culture plates at a density of  $1.8 \times 10^6$  cells/ml. Cells were incubated at 37°C in 5% CO<sub>2</sub>, and cytosine arabinoside was added 24h later to inhibit the proliferation of non-neuronal constituents. On the seventh day in vitro (DIV), glucose (50  $\mu$ l of a 100mM solution) was added to each culture well to maintain survival, as culture medium was not replaced, and

cultures were used on 8 DIV unless otherwise specified. This method yields cultures consisting of approximately 95% cerebellar granule neurons (Jiang et al., 2003).

### **3.2.3 Inhibition Studies**

For anti-NGF studies, cells were subcultured on Poly-lysine-coated, (PL; 40 µg/ml; BD Biosciences, Mississauga ON) 12-well plates under serum-starved conditions in the presence or absence of either anti-NGF (5 µM) or a control IgG (5 µM) concomitant to stimulation with exogenous NGF (50 ng/ml; Harlan, Indianapolis IN) for 24 h.

For pharmacological inhibition studies, cells were similarly subcultured on PL-coated 12-well plates under serum-starved conditions. The various pharmacological inhibitors, K252a (200 nM, Calbiochem, La Jolla CA), UO126 (10 µM; Calbiochem), LY294002 (10 µM; Calbiochem), U73343 (5 µM; Calbiochem), U73122 (5 µM; Calbiochem), or rottlerin (5 µM; Sigma, St. Louis MO) were separately added to individual wells 30 min post-plating. Inhibitor concentrations were empirically determined to provide the required inhibition (Fig 3.3A, 3.3D, 3.4C) with no detriment to viability (Fig.3.8) (Dodge et al., 2002; Mearow et al., 2002). NGF stimulation was initiated by the addition of NGF (50 ng/ml) to culture medium 2 h following introduction of the inhibitor. Cells were then allowed to incubate for 24 h at 37°C in 5% CO<sub>2</sub>.

### **3.2.4 Small Interfering RNA constructs and transfection**

SiRNA constructs were synthesized commercially (Dharmacon, Lafayette CO) based on previously reported target sequences specific for rat PKC-δ: 5'-UGA CAA GAU UAU CGG CCG CTT-3' and 5'-GCG GCC GAU AAU CUU GUC ATT-3'(Irie et al., 2002).



PC12 cells were transfected with 250 nM of PKC- $\delta$  siRNA using the Amaxa electroporation system according to manufacturer's protocols (Cell Line Nucleofector kit, program U-29). The siRNA transfection efficiency was assessed to be >70% based on transfection of Alexa fluor-labelled negative control siRNA (scrambled; target sequence AATTCTCCGAACGTGTCACGT; Qiagen, Mississauga ON). Following transfection, cells were washed in RPMI and cultured on PL-coated 12-well culture plates under reduced serum conditions for 48 h at 37°C, 5% CO<sub>2</sub>, prior to the addition of NGF (50ng/ml). Protein was harvested 72 h following electroporation, 24 h after the addition of NGF, and prepared for Western blotting.

PKC- $\delta$  siRNA was introduced to the cerebellar granule neurons at 6DIV, using a calcium phosphate coprecipitation method as previously described (Bingham et al., 2006). Briefly, conditioned medium was removed and saved for replacement later. Cells were washed twice with transfection medium (DMEM supplemented with 25mM KCl). A calcium phosphate coprecipitate of siRNA oligos was prepared and applied to the cells dropwise. Following an incubation of 2 h, cells were washed twice with transfection media and returned to incubate in the conditioned media previously removed. At 8DIV, cells were stimulated with BDNF and left 72 h post stimulation prior to collection for Western blotting.

### **3.2.5 Western Blot Analysis**

For Western analyses, cells were harvested in the presence of sodium orthovanadate (100 mM in TBS) and subsequently subjected to lysis (10% glycerol, 1% NP-40, sodium vanadate, sodium fluoride, sodium dodecyl sulphate (SDS) and 1 protease inhibitor



cocktail tablet (Roche Scientific, Laval, QC)) overnight at 4° C prior to centrifugation (10,000 g, 5 min). A BCA protein assay (Pierce, Rockford IL) was used to determine protein concentration and equivalent amounts of protein (50 µg) were electrophoresed on 8% SDS-polyacrylamide gels. Protein was subsequently transferred to nitrocellulose membranes that were then exposed to Ponceau red to ensure equal protein loading. After washing in TBS, blots were blocked in 3% non-fat dry milk for 1 h at room temperature, and then incubated with a primary antibody directed toward either p75NTR (clone MC-192; GR10; Oncogene, San Diego CA) or mitogen-activated protein kinase (MAPK; sc-94; Santa Cruz) overnight at 4° C. A final incubation with HRP-conjugated secondary antibody (AP307P, AP308P; Chemicon, Temecula, CA) for 1 h at room temperature was followed by visualization using an ECL method. Each experiment was repeated three times, subjected to densitometry and normalized to mitogen-activated protein kinase (MAPK) to ensure equal protein for comparison. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego CA) with significance being determined using one-way ANOVA testing.

### **3.2.6 Real-time RT-PCR**

For real time RT-PCR analysis, cells were subcultured on PL-coated 12-well plates under serum-starved conditions for 24 h in the presence or absence of NGF (50 ng/ml) or Brain Derived Neurotrophic Factor (BDNF; 25 ng/ml; PeproTech, Inc., Rocky Hill, NJ). For inhibition studies, PLC-γ inhibitor U73122 (5 µM; Calbiochem), or its inactive control analog U73343 (5 µM; Calbiochem) was added to cells 30 min post plating, and 2 h prior to stimulation with NGF (50 ng/ml). RNA was isolated using Trizol reagent (Invitrogen)

as per the manufacturer's instructions and was subsequently treated with DNase (Ambion; Austin TX) to remove traces of contaminating DNA. RNA (2 µg) was reverse transcribed to cDNA using MMLV reverse transcriptase (200 U for 30 min at 37 °C; Invitrogen) prior to use as template for real-time PCR amplification using the following PCR primer pairs: p75NTR forward 5'-TGCATCTGAGCTGGTGTCTGTCTT, p75NTR reverse 5'-TGCGTACAATGCTCCTGGTCTCTT, GAPDH forward 5'-CCATCACCATCTTCCAGGAG, GAPDH reverse 5'-CCTGCTTCACCACCTTCTTG, 28S forward 5'-GACCAAGGAGTCTAACGC, 28S reverse 5'-GTACGCTCGTGCTCCA. PCR amplification was performed using the Roche LightCycler (Roche) and quantified using SYBR green I. p75NTR mRNA expression was subsequently normalized using the housekeeping gene, GAPDH or 28S as indicated.

### **3.2.7 Cellular viability assay**

Cell survival in the presence of the various pharmacological inhibitors was determined based on the metabolic conversion of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to a formazan product, as described previously (Dodge et al., 2002; Mearow et al., 2002). Briefly, cells were subcultured in a PL-coated 96-well plate, and exposed to the pharmacological inhibitors in the medium for 24 h at the following concentrations: K252a (200 nM), UO126 (10 µM), LY294002 (10 µM), U73343 (5 µM), U73122 (5 µM), or rottlerin (5 µM). After 24 h media was aspirated, replaced with MTT (1 mg/ml in PBS) and allowed to incubate for 4 h at 37°C. The reaction was stopped by aspirating the MTT solution and adding DMSO (Sigma) to



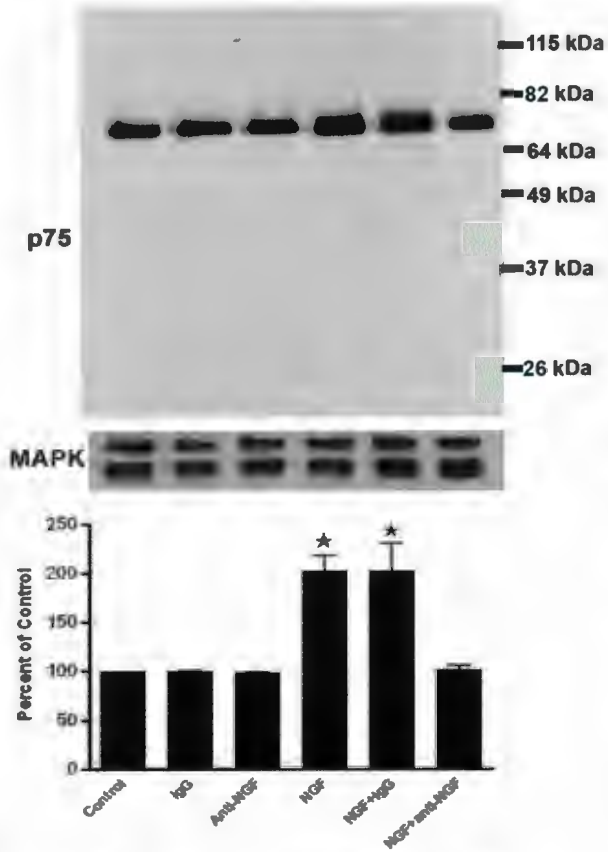
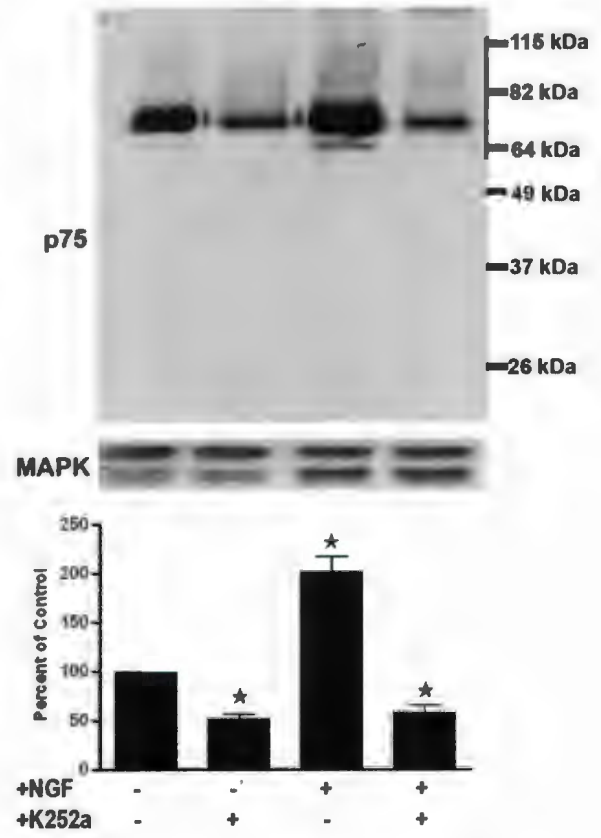
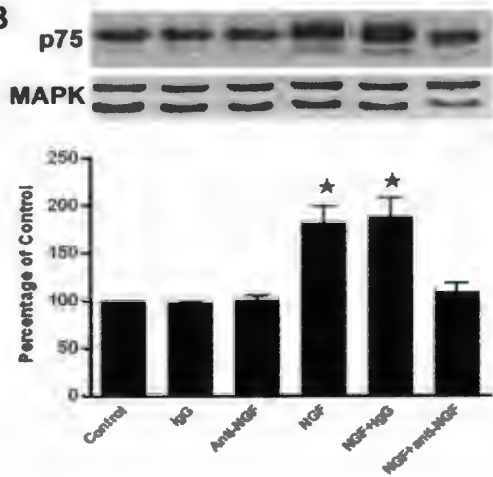
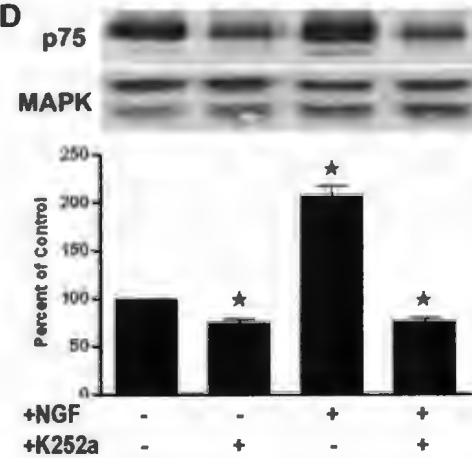
lyse the cells and solubilize the blue formazan product, which results from the mitochondrial metabolism of MTT in viable cells. After 10 min, relative quantities of formazan product were determined spectrophotometrically using a wavelength 570 nm and reference wavelength of 630 nm. Percent survival was calculated as a comparison with untreated control cells grown in the absence of inhibitors.  $\text{CoCl}_2$  (150  $\mu\text{M}$ ; Sigma) added to the cells 30 min prior to the addition of MTT served as a positive control to validate the assay. Statistical analysis was performed using GraphPad Prism 4 with significance being determined using one-way ANOVA testing.

### 3.3 Results

Our previous work indicated that the individual phosphorylation sites on the cytoplasmic tail of TrkA could alter the cellular response to NGF, including the upregulation of p75NTR expression (Rankin et al., 2005). To first ensure that exogenously supplied NGF induced this p75NTR upregulation, we incubated the cells in either anti-NGF (5  $\mu\text{M}$ ) or control IgG (5  $\mu\text{M}$ ) concomitant to stimulation with NGF (50 ng/ml) for 24 h. The addition of anti-NGF or control IgG did not alter the constitutive levels of p75NTR observed under these culture conditions in any of the cell lines. Only those cell lines expressing TrkA with a functional Y785 autophosphorylation site (PC12 and Y490F cell lines) responded to NGF by upregulating p75NTR (Fig 3.1A-PC12 cells; 3.1B-Y490F cells,  $p < 0.01$ ). In the PC12 and Y490F cell lines, the addition of anti-NGF completely abolished the upregulation of p75NTR in response to NGF, while the control IgG had no effect (Fig 3.1 A-PC12 cells, 3.1B-Y490F cells). None of the other cell lines





**A****C****B****D****Figure 3.1**

(PC12nnr5, Y785F, Y490/785F) showed any alteration in p75NTR expression in response to NGF nor showed any significant alterations in response to anti-NGF or control IgG.

PC12 cells and mutated derivatives co-express TrkA and p75NTR, both of which could potentially transduce signals following NGF binding. In order to confirm TrkA-mediated NGF signalling was responsible for altered p75NTR expression, we utilized a selective inhibitor of TrkA, K252a, to inhibit the activity of TrkA in response to NGF (Nakanishi et al., 1995). Cells were pre-incubated with K252a (200 nM) for 2 h prior to the addition of NGF (50 ng/ml). The addition of K252a reduced constitutive p75NTR levels in PC12 cells (Fig 3.1C,  $p < 0.01$ ), as well as in mutated derivatives expressing the TrkA Y490F (Fig 3.1D,  $p < 0.01$ ), to levels comparable to those seen in PC12nnr5 cells. K252a also completely prevented the NGF induced upregulation of p75NTR in PC12 (Fig 3.1C,  $p < 0.01$ ) and Y490F cell lines (Fig 3.1D,  $p < 0.01$ ). No significant effects of K252a were detected in the PC12nnr5 cell line, or the mutated derivative expressing the TrkA Y785F mutation (data not shown). The role of TrkA activation in the NGF-induced upregulation was confirmed using antisense oligos against TrkA, which similarly decreased the expression of p75NTR and prevented the upregulation of p75NTR in response to NGF (data not shown).

In order to determine if p75NTR activation contributes to an autocrine regulatory loop in response to ligand binding, p75NTR was stimulated in the absence of TrkA activation via the addition of BDNF (25 ng/ml) to the culture medium. Since PC12 cells are TrkB deficient, BDNF stimulation selectively activates p75NTR receptor signalling in the absence of TrkA activation. BDNF stimulation resulted in no significant alterations



of p75NTR expression in any of the PC12 cell lines (Fig 3.2A). In fact, BDNF stimulation of up to 200ng/ml was insufficient to upregulate p75NTR expression, although BDNF stimulation did result in the phosphorylation of Jun N-terminal kinase (JNK) at all tested concentrations (Fig 3.3).

The p75NTR receptor is known to undergo ectodomain shedding and  $\gamma$ -secretase mediated cleavage (Lee et al., 2001; Struhl and Adachi, 2000). To ensure that the differing quantities of p75NTR being detected by Western blotting were in fact due to upregulation of receptor expression, and not simply decreased detection in control samples due to ectodomain shedding, a real-time RT-PCR technique was utilized to quantitate relative amounts of p75NTR mRNA in each cell line in response to NGF stimulation (50 ng/ml), or BDNF stimulation (25 ng/ml). In response to NGF, p75NTR mRNA upregulation is noted only in the PC12 and Y490F cell lines (Fig 3.2B), confirming results obtained by Western blotting. Furthermore, BDNF stimulation had no significant effect on p75NTR mRNA expression levels in any of the cell lines (Fig 3.2C).

TrkA activation following ligand binding results in tyrosine autophosphorylation that is associated with the initiation of at least three distinct signalling cascades: the PI3K-Akt cascade, the Ras-MAPK cascade, and the PLC- $\gamma$ -PKC cascade (Loeb et al., 1994; Obermeier et al., 1993a; Obermeier et al., 1993b; Stephens et al., 1994). Since TrkA appears to be responsible for the NGF-induced upregulation of p75NTR, one of these three TrkA-associated signalling cascades may be directly responsible for mediating p75NTR upregulation. We used pharmacological inhibitors to analyze signalling intermediates to determine the relative influence of each pathway in mediating p75NTR upregulation in response to NGF.

**Figure 3.2: Selective activation of p75NTR does not contribute to ligand-induced**

**upregulation of p75NTR.** *A:* PC12 cells, PC12nnr5 cells and PC12nnr5 cells expressing TrkA with mutations Y490F, Y785F or Y490/785F were cultured in the presence or absence of BDNF (25 ng/ml) for 24 h. Since PC12 cells are TrkB deficient, BDNF activates p75NTR in the absence of TrkA activation. This selective p75NTR activation does not result in upregulation of p75NTR in any of the cell lines tested. *B,C:* Analysis of p75NTR mRNA in response to NGF or BDNF stimulation reveals differential gene transcription. PC12 cells, PC12nnr5 cells and PC12nnr5 cells expressing TrkA with mutations Y490F, Y785F or Y490/785F were cultured in the presence or absence of (*B*) NGF (50 ng/ml) or (*C*) BDNF (25 ng/ml) for 24 h. p75NTR mRNA transcripts were quantitatively determined by real-time RT-PCR analysis revealing a significant increase in p75NTR mRNA expression only in response to NGF, and only in those cell lines expressing a functional Y785 autophosphorylation site (PC12 and Y490F cell lines). Values expressed represent the mean p75NTR expression, relative to MAPK (*A*) or GAPDH (*B,C*), of 3 experiments +/- SEM. \* $p < 0.05$ , \*\* $p < 0.001$  as determined by one-way ANOVA.

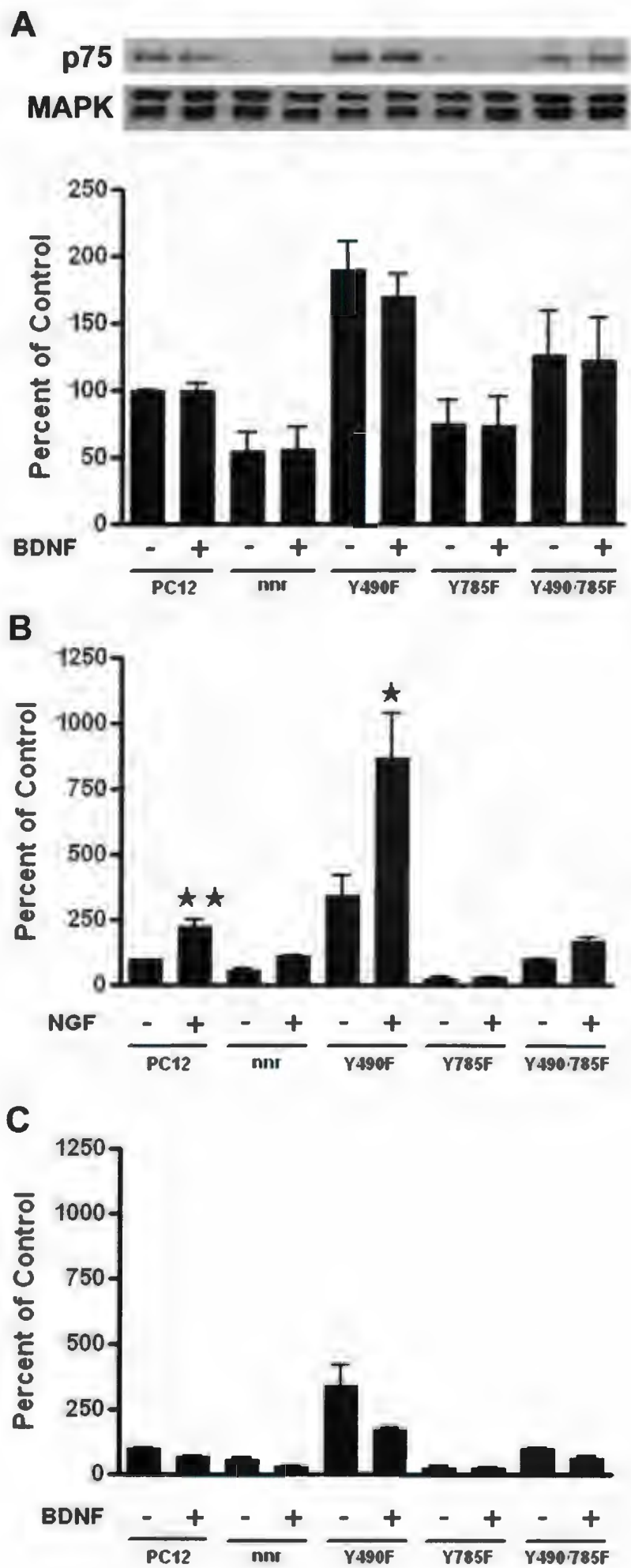


Figure 3.2



**Figure 3.3: p75NTR activation phosphorylates JNK.** PC12 cells cultured in the presence of varying concentrations of BDNF (25ng/ml, 100ng/ml, 200ng/ml) did not upregulate p75NTR in response to p75NTR activation (A), though the stimulation was sufficient to phosphorylate JNK (B). The upregulation of p75NTR in response to NGF stimulation (C) is also associated with a significant increase in phosphorylated JNK (D). \* $p < 0.05$ , \*\* $p < 0.001$  as determined by one-way ANOVA (A,B), \* $p < 0.05$ , \*\* $p < 0.005$  as determined by ttest (C,D).

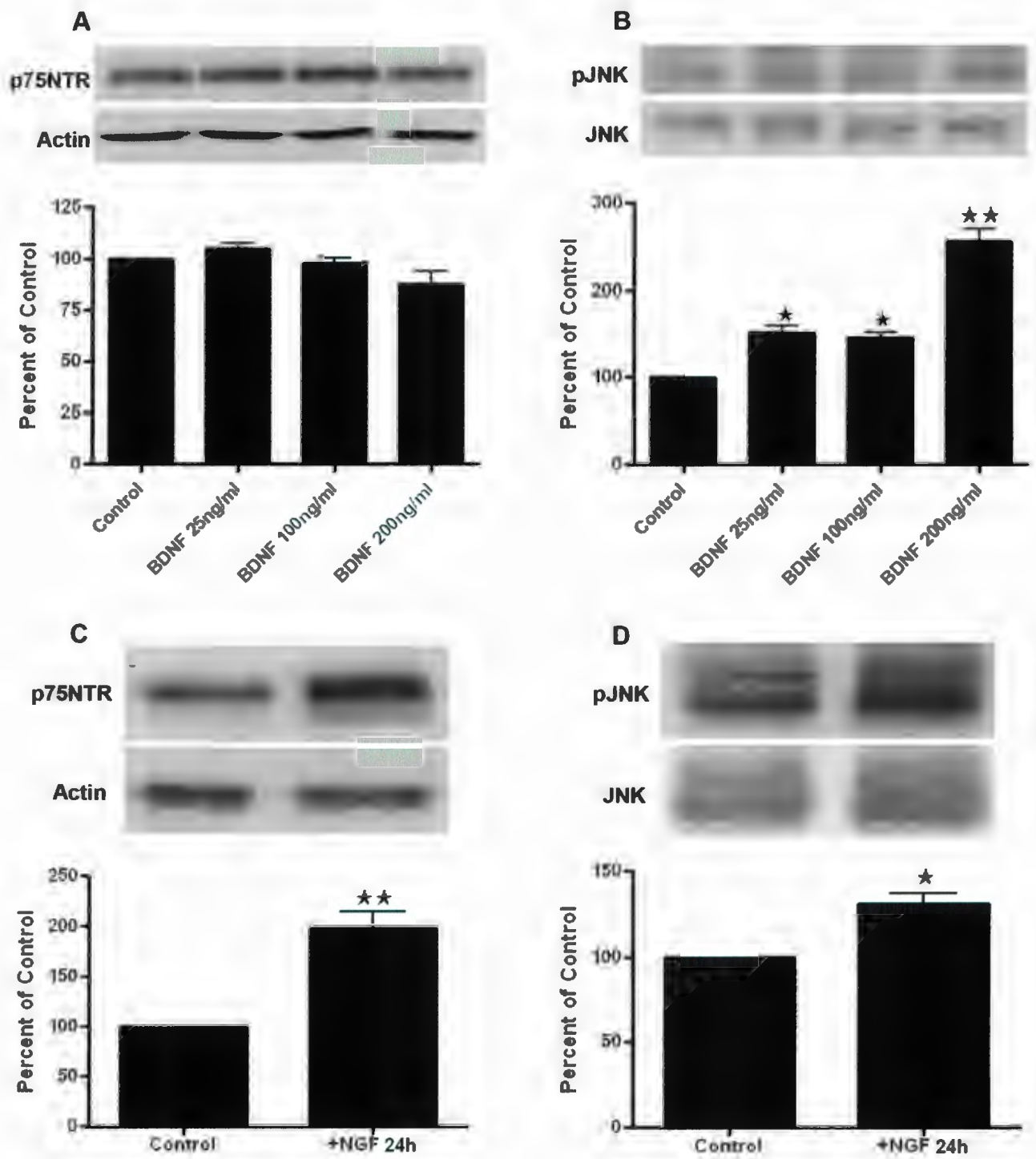


Figure 3.3

Firstly, an inhibitor of PI3K activity, LY294002 (10  $\mu$ M) was utilized to block downstream activation of intermediates in the PI3K-Akt signalling cascade. The functionality of the inhibitor was validated by a notable decrease in the phosphorylation of Akt in response to NGF at the concentration used (Fig 3.4A). The addition of LY294002 to the culture medium resulted in a small but significant decrease in constitutive p75NTR expression in PC12, Y490F and Y490/785F cell lines, consistent with previous reports (Musatov et al., 2004). However, the presence of LY294002 failed to prevent the NGF-induced upregulation of p75NTR seen in PC12 (Fig 3.4B,  $p < 0.01$ ) and Y490F cell lines (Fig 3.4C,  $p < 0.01$ ), thus indicating that PI3K pathway does not seem to play a direct role in regulating p75NTR expression.

Secondly, an inhibitor against the Y490 activated signalling cascade was utilized. UO126 inhibits MEK, as evidenced by decreased phosphorylation of downstream effector, MAPK (Fig 3.4D), thus interrupting the NGF-induced Ras-MAPK signalling cascade initiated by the binding of Shc adapter proteins to the Y490 autophosphorylation site. The addition of UO126 (10 $\mu$ M) to the culture medium resulted in a decrease in constitutive p75NTR expression in PC12 and Y490F cell lines, consistent with previous reports (Musatov et al., 2004) but again did not prevent the NGF-induced upregulation of p75NTR in PC12 (Fig 3.4E,  $p < 0.01$ ) and Y490F cell lines (Fig 3.4F,  $p < 0.01$ ). These data indicate that MAPK signalling is not required for the NGF-dependent regulation of p75NTR expression. In order to analyze the involvement of the third and final TrkA activation induced signalling cascade in the NGF-induced upregulation of p75NTR, U73122 was utilized to inhibit agonist-induced PLC-  $\gamma$  activation, thus preventing activation of downstream effectors. The inhibition caused by U73122 was contrasted



**Figure 3.4: Inhibition of PI3K or MEK activity reduces constitutive p75NTR expression, but does not prevent the NGF-induced upregulation of p75NTR.** PC12 cells (*B,E*) and PC12 cells expressing TrkA with a Y490F mutation (*C,F*) were cultured for 24 h in the presence or absence of NGF (50 ng/ml) and/or LY294002 (10  $\mu$ M), an inhibitor of PI3K activity, or UO126 (10  $\mu$ M), an inhibitor of MEK activity. LY294002 suppresses the phosphorylation of Akt (*A*). UO126 suppresses the phosphorylation of MAPK (*D*). The addition of LY294002 (*B,C*) or UO126 (*E,F*) each significantly decreased the constitutive expression of p75NTR in both cell lines, but failed to abrogate the NGF-induced upregulation of p75NTR in either. Values expressed represent the mean p75NTR protein expression, relative to MAPK, of 3 experiments  $\pm$  SEM. \* $p < 0.01$  as determined by one-way ANOVA.

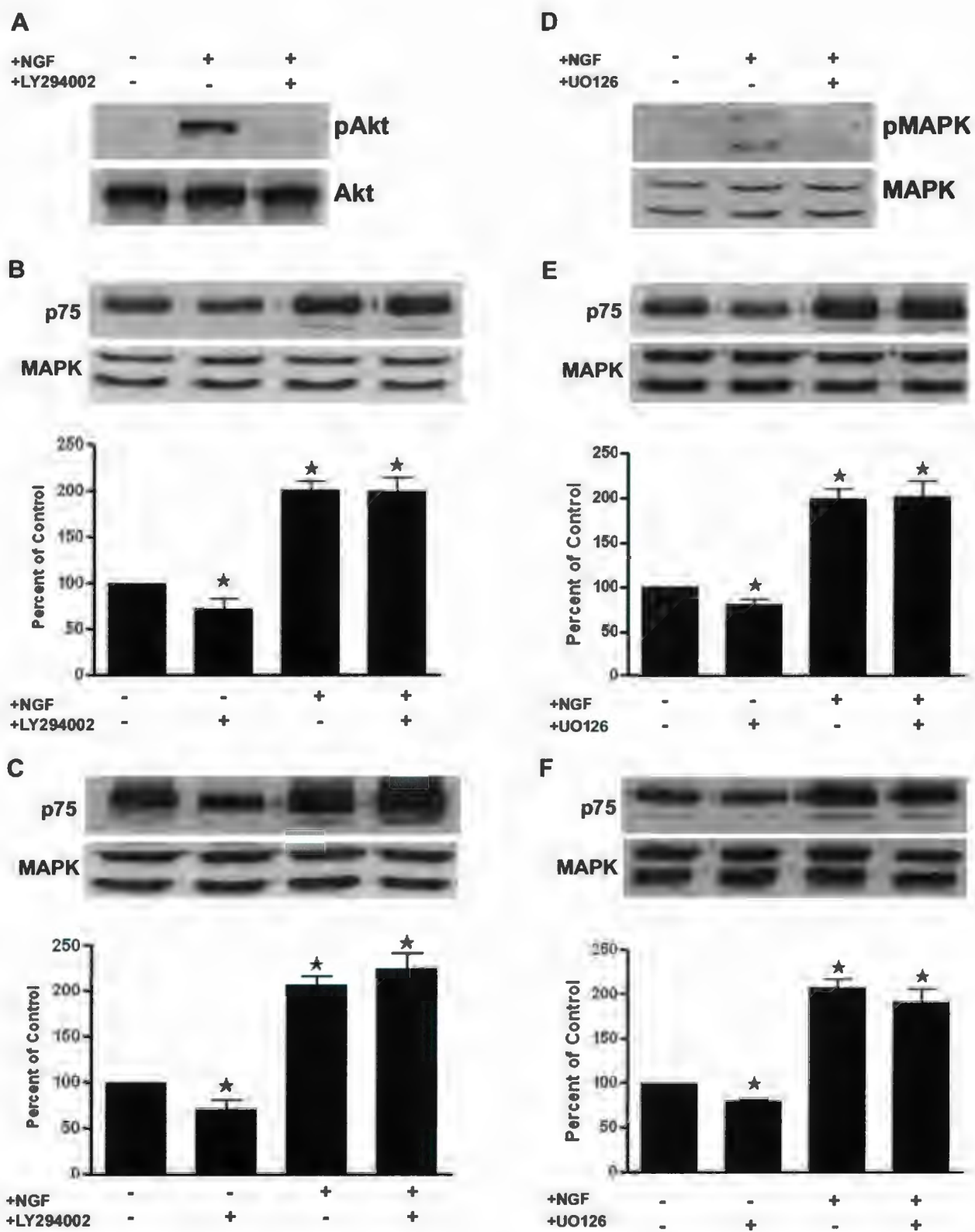


Figure 3.4

with effects caused by its inactive analog U73343. The addition of U73343 (5 $\mu$ M) had no effect on constitutive expression of p75NTR, nor did it prevent the upregulation of p75NTR in response to NGF ( $p < 0.01$ ). In contrast, the addition of U73122 completely eliminated the NGF-induced upregulation of p75NTR in both PC12 cells (Fig 3.5A) and Y490F cells (Fig 3.5B), without affecting constitutive expression.

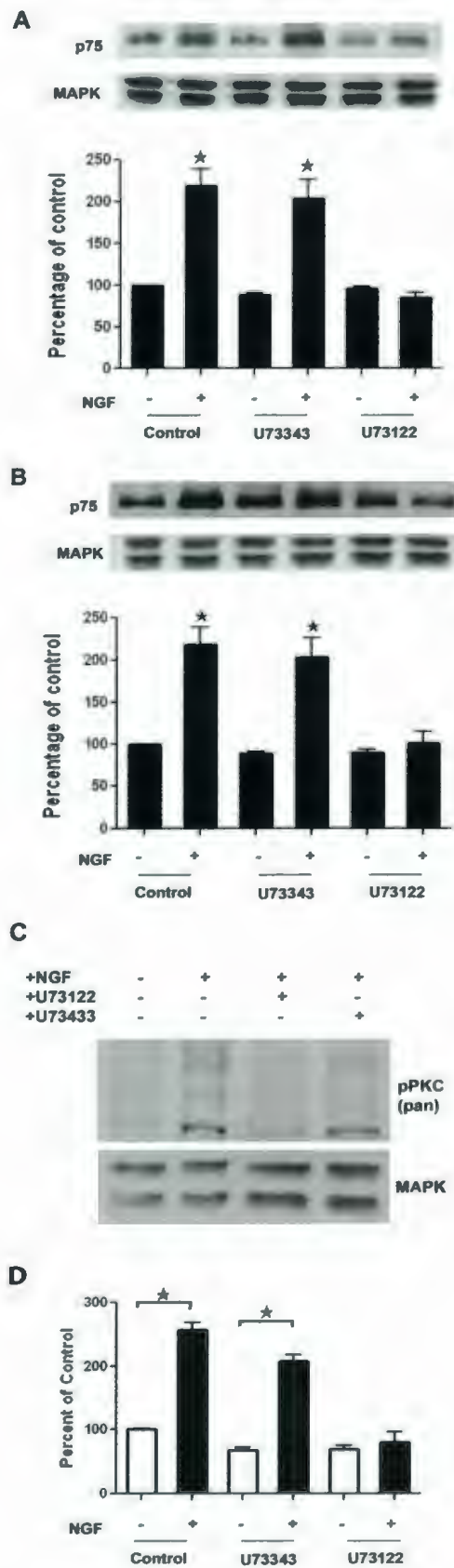
Since PKC isoforms can be activated downstream of PLC- $\gamma$ , we assessed whether PLC- $\gamma$  inhibition had any influence on PKC activation using a pan-specific phosphorylated PKC antibody. Western determination of phosphorylated pan-PKC showed an increase in response to NGF stimulation, which was significantly blocked by the presence of U73122 (Fig 3.5C).

Further evidence for the involvement of PLC- $\gamma$  activity in the upregulation of p75NTR was obtained from real-time RT-PCR studies, which confirmed that inhibition of PLC- $\gamma$  using U73122 prevented p75NTR upregulation in response to NGF stimulation in PC12 cells, while the less active analog U73343 did not (Fig. 3.5D,  $p < 0.01$ ).

PLC- $\gamma$  activation results in the downstream activation of various PKC isoforms. A previous study of osmotic insult (Peterson and Bogenmann, 2003) noted that fluctuations of p75NTR expression were partially eliminated by the addition of rottlerin, a PKC- $\delta$  inhibitor. This indicated that PKC- $\delta$  might play a regulatory role for p75NTR in the injury paradigm. Thus, to ascertain if PLC- $\gamma$  is signalling through PKC- $\delta$  to regulate p75NTR upregulation in response to neurotrophin stimulation, we investigated the effect of rottlerin treatment. The addition of rottlerin (5  $\mu$ M) to culture medium for 24 h completely prevented the upregulation of p75NTR in response to NGF stimulation in both PC12 cells (Fig. 3.6A), and Y490F cells (Fig. 3.6B).



**Figure 3.5: Inhibition of PLC- $\gamma$  blocks the NGF-induced upregulation of p75NTR protein and mRNA transcription.** PC12 cells (*A*) and PC12 cells expressing TrkA with a Y490F mutation (*B*) were cultured in the presence or absence of NGF (50 ng/ml) and/or PLC- $\gamma$  inhibitor, U73122 (5  $\mu$ M), or its inactive analogue U73343 (5  $\mu$ M) for 24 h. The addition of U73343 had no significant effect on the constitutive expression of p75NTR, nor its upregulation in response to NGF stimulation. In contrast, the addition of U73122 completely prevented the NGF-induced upregulation of p75NTR, thus demonstrating that p75NTR upregulation occurs via a PLC- $\gamma$  dependent mechanism. Similar results were noted for mRNA transcripts quantified using real-time RT-PCR (*D*). The parallel result of mRNA to protein under these culture conditions eliminates the possibility that ectodomain shedding is responsible for reducing detectability of p75NTR. Values expressed represent the mean p75NTR protein expression, relative to MAPK (*A,B*) or 28S (*D*), of 3 experiments  $\pm$  SEM. \* $p < 0.01$  as determined by one-way ANOVA. PLC- $\gamma$  activation results in the phosphorylation of several PKC isoforms detectible using an antibody directed against pan-phospho-PKC (bands shown range from 78 kDa to 116 kDa). This phosphorylation is notably decreased in the presence of U73122, but not in the presence of U73343 (*C*).



**Figure 3.5**

**Figure 3.6: Inhibition of PKC- $\delta$  prevents the NGF-induced upregulation of p75NTR.** PC12 cells (*A*) and PC12 cells expressing TrkA with a Y490F mutation (*B*) were cultured in the presence or absence of NGF (50 ng/ml) and/or PKC- $\delta$  inhibitor, rottlerin (5  $\mu$ M) for 24 h. The addition of rottlerin completely prevented the NGF induced upregulation of p75NTR without influencing the constitutive expression, thus implicating PKC- $\delta$  isoforms in the upregulation of p75NTR. A more specific means of interfering with the action of PKC-  $\delta$ , transfection with siRNA constructs specific to PKC-  $\delta$ , successfully decreased the expression of PKC- $\delta$  48 h following transfection (*C*). This decrease in PKC-  $\delta$  effectively prevented the NGF-induced upregulation of p75NTR while a scramble control siRNA sequence had no effect (*D*). Values expressed represent the mean p75NTR protein expression, relative to MAPK (*A,B*), or actin (*C,D*), of 3 experiments  $\pm$  SEM. \* $p < 0.05$  as determined by one-way ANOVA.



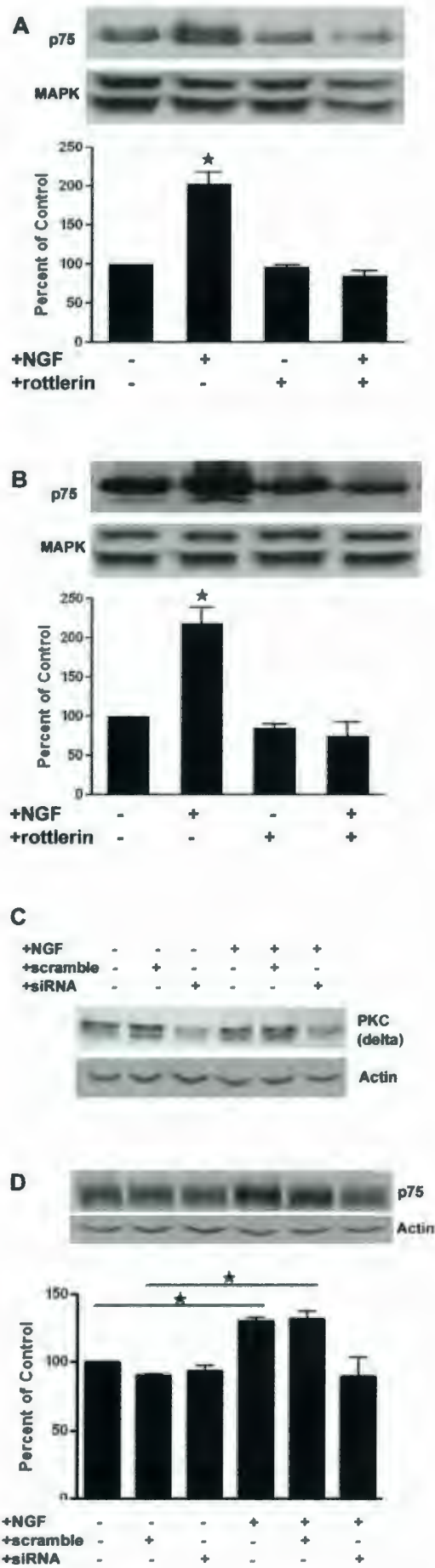


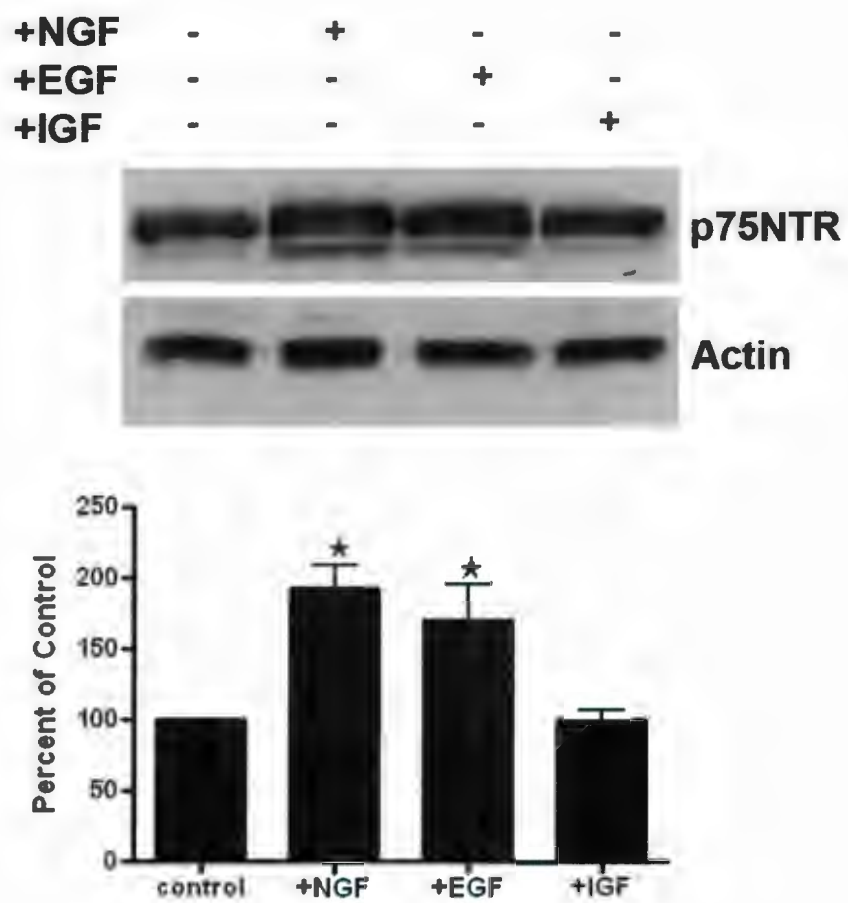
Figure 3.6

We then examined the involvement of PKC- $\delta$  in the upregulation of p75NTR expression, by employing a more specific means of inhibition in the form of small-interfering RNA (siRNA). siRNA oligos specific to rat PKC- $\delta$  were designed based on previously published sequences (Irie et al., 2002), and PC12 cells were transfected using nucleoporation. 48 h following transfection, cells were stimulated with NGF (50 ng/ml) for a further 24 h prior to protein collection. The introduction of siRNA constructs to PC12 cells efficiently decreased expression of PKC- $\delta$  over the experimental timeframe, while negative control siRNA constructs (scrambled) had no effect on PKC- $\delta$  expression (Fig. 3.6C). NGF induced a significant upregulation of p75NTR in the presence of the scrambled construct ( $p < 0.05$ ), which is abolished in the presence of PKC- $\delta$  siRNA constructs (Fig. 3.6D). Although the magnitude of NGF-induced upregulation of p75NTR was lower following electroporation (Fig. 3.6D) than is noted in other figures, we have consistently observed that electroporation of PC12 cells reduces their capacity to upregulate p75NTR expression. Nonetheless, these data establish a role for PKC- $\delta$  in the regulation of p75NTR expression.

Further evidence for the involvement of the PLC $\gamma$ -PKC $\delta$  signalling intermediates in the regulation of p75NTR expression comes from the activation of this signalling cascade by a means alternative to TrkA activation. The addition of epidermal growth factor (EGF; 50ng/mL) to PC12 cell cultures for 24h activates the PLC $\gamma$ -PKC $\delta$  signalling cascade by binding to the EGF receptor (Chen et al., 1996b; Welsh et al., 1991). This ligand is also able to induce the upregulation of p75NTR, despite a lack of TrkA receptor activation (Fig 3.7,  $p < 0.05$ ). However, p75NTR upregulation is not ubiquitous to all receptor tyrosine kinase activation, as exposure to IGF1 (50ng/ml) for 24h, which does

**Figure 3.7: Stimulation of the PLC $\gamma$ -PKC- $\delta$  pathway via EGF-R or TrkA activation upregulates p75NTR expression, while selective activation of IGF-R is insufficient to alter p75NTR expression level.** PC12 cells were incubated with NGF (50 ng/ml), EGF (50 ng/ml) or IGF (50 ng/ml) for 24 h prior to collection and lysis for Western blot analysis. P75NTR was detected using an antibody directed against the intracellular domain of p75NTR (Upstate). Values expressed represent the mean p75NTR protein expression, relative to actin of 3 experiments  $\pm$  SEM. \* $p < 0.05$  as determined by one-way ANOVA.





**Figure 3.7**

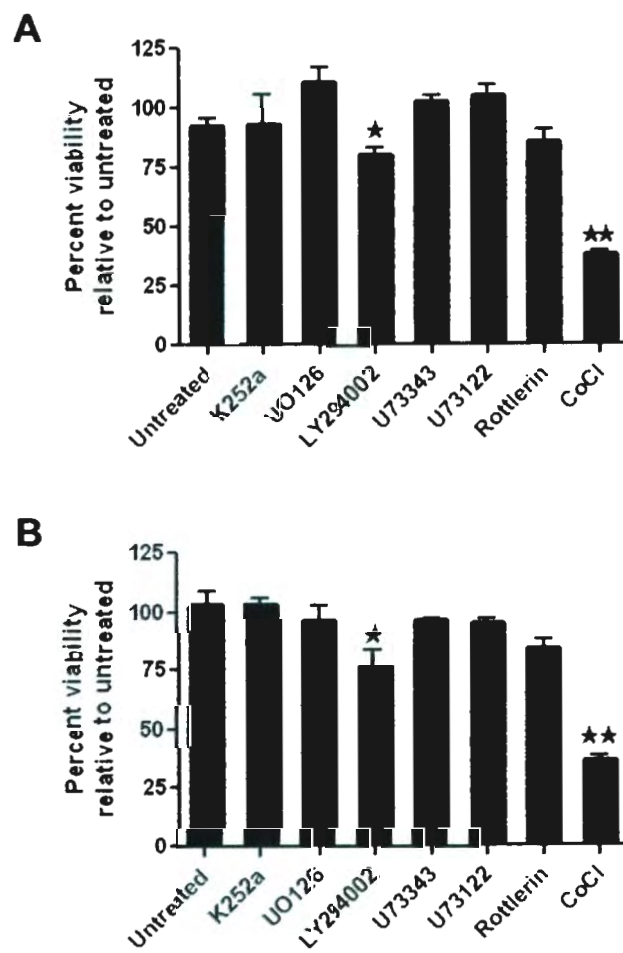
not activate PLC- $\gamma$  (Kimpinski and Mearow, 2001; Zapf-Colby et al., 1999), was insufficient to induce p75NTR upregulation (Fig 3.7).

The study of p75NTR function has revealed a prominent role for this receptor in apoptosis (reviewed in Dechant and Barde, 2002). In order to distinguish the effects of the inhibitors on p75NTR expression from their effects on cell survival, an MTT survival assay was performed to ensure that decreased cellular viability under the culture conditions was not resulting in altered p75NTR expression. Only the use of LY294002, an inhibitor of the PI3K-Akt pathway for cell survival, resulted in a slight but significant decrease in cell viability ( $p < 0.05$ ). None of the other inhibitors influenced cell viability at the concentrations used (Fig. 3.8).  $\text{CoCl}_2$  (150 $\mu\text{M}$ ) was used as a positive control since previous studies (Fleury et al., 2006; Yang et al., 2004) have demonstrated that this concentration induces cell death. Furthermore, a vehicle control (DMSO) for each inhibitor did not influence p75NTR expression or upregulation in response to NGF (data not shown).

To determine the potential functional relevance of the proposed PKC $\delta$ -dependent mechanism in primary neurons, we employed a culture model of primary cerebellar granule neurons (CGN). CGN express only TrkB, TrkC and p75NTR neurotrophin receptors (Zirrgiebel and Lindholm, 1996). Since TrkB ligation results in the activation of similar signalling pathways as TrkA, we hypothesized that BDNF exposure would result in the upregulation of p75NTR. CGN were exposed to one of NGF, BDNF or NT-3 at the time of plating, and protein was sampled following 8 DIV. As illustrated in Figure 3.9A-C, the addition of BDNF upregulated p75NTR protein expression in CGN ( $p < 0.01$ ), while NGF and NT-3 had no effect. Since CGN are TrkA-deficient, NGF

**Figure 3.8: The decreased p75NTR expression in response to pharmacological inhibition is not associated with toxicity.** Inhibitor concentrations were previously empirically determined to be the lowest effective concentration that resulted in biochemical inhibition with minimal or no cell death. PC12 cells (*A*) and PC12 cells expressing TrkA with a Y490F mutation (*B*) were cultured in the presence or absence of K252a (200 nM), UO126 (10  $\mu$ M), LY294002 (10  $\mu$ M), U73343 (5  $\mu$ M), U73122 (5  $\mu$ M) or rottlerin (5  $\mu$ M) for 24 h prior to the determination of cellular viability based on the conversion of MTT to a formazan product. Only the addition of LY294002 caused a slight but significant decrease in viability. The assay was validated by the addition of  $\text{CoCl}_2$  (150  $\mu$ M), which perturbs mitochondrial activity, to control wells. Values expressed represent the mean percentage of survival, relative to untreated cells, of 3 experiments  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.0001$  as determined by one-way ANOVA.





**Figure 3.8**

**Figure 3.9: Activation of TrkB upregulates p75NTR via a PKC-  $\delta$ -dependent mechanism in cerebellar granule neurons.** *A:* Cerebellar granule neurons were exposed to either NGF (50 ng/ml), BDNF (25 ng/ml) or NT-3 (25 ng/ml) at the time of plating and harvested following 8 DIV. The addition of NGF or NT-3 had no significant effect on the expression of p75NTR. In contrast, the addition of BDNF resulted in a dramatic upregulation of p75NTR. *B,C:* Cerebellar granule neurons were immunostained for p75NTR to show the upregulation of p75NTR in response to BDNF exposure. Note the punctate nature of the p75NTR immunostain. *D:* Cerebellar granule neurons were cultured in the presence or absence of LY294002, UO126, U73343 or U73122 and/or BDNF (25ng/ml). Only the PLC- $\gamma$  inhibitor U73122 prevented the BDNF-induced upregulation of p75NTR. *E:* Cerebellar granule neurons were plated on PL and cultured for 24h in the presence of NGF (50 ng/ml), BDNF (25 ng/ml) or NT-3 (25 ng/ml) prior to protein collection and lysis for Western blot analysis using antibodies directed against phosphorylated PKC- $\delta$  and PKC- $\delta$ . Stimulation of cerebellar granule neurons with BDNF, but not NGF or NT-3, results in the phosphorylation of PKC- $\delta$ . *F,G:* The transfection with siRNA constructs specific to PKC-  $\delta$  successfully decreased the expression of PKC- $\delta$ , and this decrease in PKC-  $\delta$  effectively prevented the BDNF-induced upregulation of p75NTR while a scramble control siRNA sequence had no effect. Values expressed represent the mean phosphorylated PKC- $\delta$  protein expression, relative to PKC of 3 experiments  $\pm$  SEM. \* $p < 0.01$  as determined by one-way ANOVA.

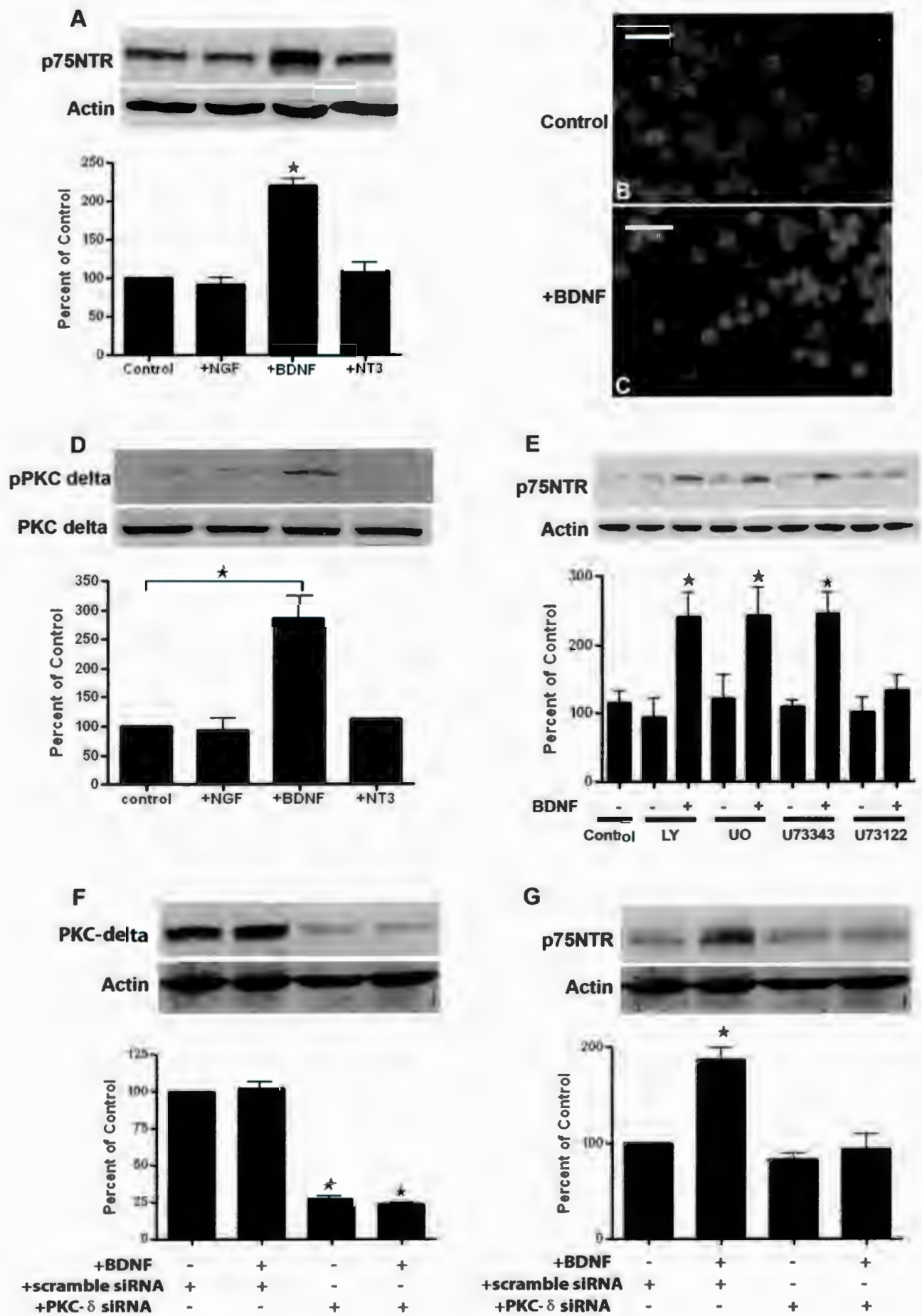


Figure 3.9



would bind only p75NTR in these cells, and as shown in Fig. 3.2, p75NTR stimulation is not sufficient for its upregulation. NT-3 binding TrkC was also insufficient to upregulate p75NTR, but past studies have highlighted the differences in activation of intracellular molecules by BDNF versus NT-3, including the inability of NT-3 to activate the PLC- $\gamma$  signalling cascade in cerebellar granule neurons (Zirrgiebel and Lindholm, 1996). We confirmed the inability of NT-3 stimulation to phosphorylate PKC- $\delta$  (Fig. 3.9D,  $p < 0.01$ ).

To confirm that the BDNF-TrkB signalling for the upregulation of p75NTR was occurring via the same mechanism as the NGF-TrkA signalling, cerebellar granule neurons were exposed to a similar panel of pharmacological inhibitors. Inhibition of the PI3K cascade using LY294002 was insufficient to prevent the BDNF-induced upregulation of p75NTR, as was inhibition of the MEK-MAPK pathway using UO126. Conversely, treatment with the PLC- $\gamma$  inhibitor U73122 was able to prevent the BDNF-induced upregulation of p75NTR in cerebellar granule neurons (Fig. 3.9E).

In order to determine if this BDNF-induced upregulation of p75NTR was also occurring via a PKC- $\delta$  dependent mechanism, we once again employed the specific PKC- $\delta$  siRNA oligos to suppress expression of PKC- $\delta$ . The siRNA was introduced to the cerebellar granule neurons using a calcium phosphate coprecipitation. 48h following transfection, cells were stimulated with BDNF (25 ng/ml) for a further 72 h prior to protein collection. The introduction of siRNA constructs to cerebellar granule neurons efficiently decreased expression of PKC- $\delta$  over the experimental timeframe ( $p < 0.01$ ), while negative control siRNA constructs (scrambled) had no effect on PKC- $\delta$  expression (Fig. 3.9F). BDNF induces a significant upregulation of p75NTR in the presence of the scrambled construct ( $p < 0.01$ ), which is abolished in the presence of PKC- $\delta$  siRNA (Fig.

3.9G). These data confirm a role for PKC- $\delta$  in the regulation of p75NTR expression in cerebellar granule neurons.

### 3.4 Discussion

p75NTR expression levels play a central role in the regulation of neuronal survival and axonal growth in response to target-derived neurotrophins, and subsequently regulate innervation density in the developing nervous system. Elucidation of the mechanism regulating p75NTR upregulation provides insight into the regulation of these essential biological processes.

We have utilized a well characterized model, consisting of PC12 cells and derivatives expressing a TrkA receptor mutated to abrogate either one or both intracellular autophosphorylation sites (Loeb et al., 1994; Obermeier et al., 1993a; Obermeier et al., 1993b; Stephens et al., 1994), to investigate the molecular mechanism behind the neurotrophin-induced upregulation of p75NTR. PC12nnr5 cells are TrkA deficient and are thus devoid of classical NGF responses, including the induction of neurite outgrowth and neuronal differentiation (Loeb et al., 1991). These mutants were originally characterized as sharing most of the phenotypic properties expressed by the parental cell line in the absence of NGF (Green et al., 1986). Transfection of PC12nnr5 cells with the mutated TrkA receptors partially restored NGF responses and allowed for the molecular dissection of TrkA signalling pathways (Loeb and Greene, 1993).

TrkA activation is known to initiate three distinct signalling cascades: Y490 serves as a docking site for Shc to initiate the Ras-MAPK cascade, and also binds



adapters FRS2 and FRS3, and Y785 serves as an interaction site for PLC- $\gamma$  to initiate a PKC-MAPK cascade. Additionally, the PI3K/Akt cascade for neuronal survival is activated by TrkA:NGF binding (Dixon et al., 2006; Loeb et al., 1994; Meakin et al., 1999; Obermeier et al., 1993a; Obermeier et al., 1993b; Stephens et al., 1994). We have previously demonstrated that only those cells expressing TrkA with a functional Y785 autophosphorylation site (eg. PC12, Y490F cells) respond to NGF stimulation with an upregulation of p75NTR expression (Rankin et al., 2005), and that this is independent of TrkA expression changes. In the present study, we show that this upregulation is occurring via activation of TrkA, not p75NTR, as p75NTR activation itself is insufficient to alter its expression in response to receptor stimulation. Furthermore, we now directly implicate downstream targets of the Y785 signalling pathway, specifically PKC- $\delta$ . Finally we illustrate the relevance of this mechanism to primary cerebellar granule neurons and show that a similar mechanism exists for TrkB and BDNF regulation of p75NTR in granule neurons.

An investigation of individual TrkA-mediated signalling intermediates in each of the three cascades was performed using pharmacological inhibition, and revealed no effects of the Y490-Ras-MAPK pathway, or the PI3K/Akt pathway on NGF-induced upregulation of p75NTR. In contrast, inhibition of PLC- $\gamma$  or PKC- $\delta$ , both intermediates of the Y785-initiated cascade, prevented the upregulation of p75NTR in response to NGF stimulation. These results indicate a PLC- $\gamma$ -PKC- $\delta$  -dependent mechanism in the control of p75NTR upregulation.

p75NTR is normally expressed during developmental stages of the vertebrate nervous system, but dramatic upregulation has been reported in the period just preceding



target innervation, marking the transition to NGF-dependence. This upregulation correlates with a period of naturally occurring cell death, during which those neurons that were unsuccessful in obtaining target territory with sufficient trophic support are eliminated apoptotically (Bamji et al., 1998; Davies, 2003; Kohn et al., 1999; Nykjaer et al., 2005; Wyatt and Davies, 1993). p75NTR is also upregulated in the nervous system under certain pathological conditions of nerve injury or stress (reviewed in Dechant and Barde, 2002; Nykjaer et al., 2005), and is associated with neuronal degeneration. Interestingly, a PLC- $\gamma$ -dependent mechanism has been implicated in the upregulation of p75NTR in response to a cellular swelling insult (Peterson and Bogenmann, 2003), highlighting a potentially universal mechanism of p75NTR control.

A link between p75NTR upregulation and neuronal death has been established (Dechant and Barde, 2002), but that does not preclude the hypothesis that the regenerative response following injury or insult requires recapitulation of specific events that occur during development (Wood et al., 1990). The expression of p75NTR is not always associated with neuronal apoptosis; modulation of developmental innervation density and axonal pathfinding in the peripheral nervous system are also thought to be regulated by a p75NTR contribution. p75NTR may in fact enhance or prolong the signalling of the Trk receptor (Epa et al., 2004).

Various populations of sympathetic and sensory neurons constitutively express p75NTR throughout life in combination with expression of one or more of the Trk receptors, and exposure to NGF can upregulate p75NTR expression in adult dorsal root ganglion neurons (Lindsay et al., 1990), PC12 cells (Doherty et al., 1988), sympathetic neurons (Miller et al., 1991), and adult basal forebrain cholinergic neurons (Cavicchioli et

al., 1989). We now report that BDNF can upregulate p75NTR in CGN, using a similar PLC- $\gamma$ –PKC- $\delta$ -dependent mechanism. BDNF is expressed in the developing cerebellum, with increased levels detectable in the postnatal period (Rocamora et al., 1993), and studies using mice deficient in BDNF have revealed an essential role for this neurotrophin in the survival, maturation, and motility of CGNs postnatally (Borghesani et al., 2002; Schwartz et al., 1997). Similarly, p75NTR activity plays a notable role in the development of cerebellar foliation patterning, with pronounced defects when endogenous BDNF is also reduced (Carter et al., 2003).

Most neurons possess a complex dual growth control mechanism where 2 different transmembrane receptors share the same ligand with potentially different biological outcomes. p75NTR can signal independently of TrkA, activating sphingomyelinase (Dobrowsky et al., 1994), NF $\kappa$ B (Carter et al., 1996) and JNK (Twiss et al., 1998) signaling pathways, and activation can frequently result in apoptosis (Coulson et al., 1999). When coexpressed, p75NTR is known to cooperate with TrkA, regulating the activity of the Trk receptor (Epa et al., 2004; Roux and Barker, 2002; Zaccaro et al., 2001), enhancing the internalization of NGF (Twiss et al., 1998), and increasing the ligand specificity of TrkA (Brennan et al., 1999; Zaccaro et al., 2001). Thus, cellular responsiveness to NGF is at least partially determined, not only by the presence of p75NTR, but by the ratio of p75NTR:TrkA (Gatzinsky et al., 2001; Miller et al., 1994; Twiss et al., 1998; Yan et al., 2002). Regulation of this ratio seems to depend more upon alterations in p75NTR than TrkA (Miller et al., 1994), and in fact, the balance between p75NTR and Trk activities is essential for normal physiological function (Miller and Kaplan, 1998).

In PC12 cells, the ratio of p75NTR:TrkA is approximately 10-20:1 (Meakin and Shooter, 1992), which may be important in determining the relative activation of the 2 alternative signaling pathways triggered by NGF, and thus the biological outcome of neurotrophin exposure (Gatzinsky et al., 2001; Yan et al., 2002). An appropriate p75NTR:TrkA ratio might play a protective role (Althaus and Kloppner, 2006) and allow a silencing of p75NTR-induced cell death (Majdan et al., 2001), but could also regulate the number of high affinity binding sites (Wehrman et al., 2007).

Several studies have elucidated a role for p75NTR modulation in the homeostatic control of NGF signaling within a fluctuating neurotrophin environment (Twiss et al., 1998). The ability of a neuron to respond to neurotrophins can be intricately refined through regulation of receptor expression on the cell surface, and the binding affinity can be altered simply by fine-tuning the ratio of expression of p75NTR:TrkA (Esposito et al., 2001) to adjust cellular response to context (Blochl and Blochl, 2007). The upregulation of p75NTR in response to neurotrophins may be necessary for p75NTR to participate in a broad range of physiological functions, including axonal elongation or migration, in cellular context specific situations.



## **Chapter 4: Neurite Outgrowth is Enhanced by Laminin-Mediated Downregulation of the Low Affinity Neurotrophin Receptor, p75NTR.**

*A portion of this study has been accepted for publication in Journal of Neurochemistry, August 2008, in press.*

### **4.0 Summary**

Laminin, an extracellular matrix (ECM) component, is a key factor in promoting axonal regeneration, coordinately regulating growth in conjunction with trophic signals provided by the neurotrophins, including nerve growth factor (NGF). This study investigated potential interactions between the LN and NGF-mediated signaling pathways in PC12 cells and primary neurons. Neurite outgrowth stimulated by NGF was enhanced on a LN substrate. Western blot analysis of pertinent signal transduction components revealed both enhanced phosphorylation of early signaling intermediates upon co-stimulation, and a LN-induced downregulation of p75NTR which could be prevented by the addition of integrin inhibitory RGD peptides. This p75NTR downregulation was associated with a LN-mediated upregulation of PTEN and resulted in a decrease in Rho activity. Studies using overexpression or siRNA-mediated knock-down of PTEN demonstrate a consistent inverse relationship with p75NTR, and the overexpression of p75NTR impaired neurite outgrowth on a LN substrate, as well as resulting in sustained activation of Rho which is inhibitory to neurite outgrowth. p75NTR is documented for its role in the transduction of inhibitory myelin-derived signals, and our results point to ECM regulation of p75NTR as a potential mechanism to ameliorate inhibitory signaling leading to optimized neurite outgrowth.

#### 4.1 Introduction

The extracellular matrix (ECM) is a key factor in the promotion of axonal regeneration following nerve injury. The interaction of the extending axon with a permissive microenvironment can increase the probability of axonal initiation, rate of elongation and degree of branching (reviewed in Lemons and Condic, 2008). It is generally accepted that central nervous system (CNS) regeneration does not occur readily due in part to the non-permissive growth environment and the lack of appropriate growth factors. In contrast, regeneration occurs quite readily in the peripheral nervous system (PNS). An elegant study by David and Aguayo (David and Aguayo, 1981) highlighted the importance of ECM cues by showing that a CNS neuron could regenerate when placed in a PNS-like environment. Laminin (LN) is the most abundant component of the peripheral ECM, is upregulated following peripheral nerve injury (Wallquist et al., 2002), and likely plays a physiological role in regeneration potentially by engaging a combination of enhanced growth programs and overriding inhibitory cues. The interaction between the ECM and the cell surface is mediated by a group of receptors known as integrins. When activated by specific ligands, integrins cluster within the cell membrane forming focal aggregations that initiate various signaling cascades (Wozniak et al., 2004). The overexpression of  $\alpha 1$  or  $\alpha 5$  integrin subunits can rescue the regenerative abilities of adult CNS neurons to embryonic levels in the presence of appropriate growth promoting factors (Condic, 2001). In addition, integrins can regulate the cellular response to growth factors, including platelet-derived growth factor (Miranti and Brugge, 2002; Schwartz and Ginsberg, 2002; Yamada and Even-Ram, 2002) and epidermal growth factor (Cabodi et

al., 2004). Conversely, growth factors have been reported to modulate integrin signaling as well (Staniszewska et al., 2008).

Another key factor involved in optimal axonal regeneration following damage is the trophic support provided by neurotrophins, including nerve growth factor (NGF), which stimulates the initiation and extension of neurites by binding to receptor TrkA, and thus activating various signaling cascades (Reichardt, 2006). TrkA is a receptor tyrosine kinase that has only 2 phosphorylatable tyrosine residues on the cytoplasmic tail outside of the activation loop. These tyrosine residues have been linked to individual signaling cascades: Y490 phosphorylation activates the Shc-Ras-MAPK pathway, while Y785 phosphorylation activates the PLC $\gamma$ -PKC pathway. These signaling pathways converge to phosphorylate the mitogen-activated protein kinases (MAPK), ERK1 and ERK2 (Stephens et al., 1994).

NGF can also bind to the low affinity receptor, p75NTR, which has multiple functions ranging from apoptosis to survival depending on the availability of co-receptors and intracellular signaling partners (Blochl and Blochl, 2007). Unliganded p75NTR is known to constitutively activate RhoA, a small GTPase whose signaling is inhibitory to growth; the binding of NGF is thought to negate this activation and thus permit the extension of neurites (Yamashita et al., 1999). Furthermore, p75NTR is known to act as part of a signaling complex that includes Nogo-R and LINGO, which functions in the transduction of myelin-derived inhibitory signals (Domeniconi et al., 2005). Alternatively, p75NTR has been shown to play a role in the enhancement of TrkA activation and signaling (Epa et al., 2004; Roux and Barker, 2002).



NGF and LN signaling has been studied extensively in various models (Liu et al., 2002; Rankin et al., 2006; Tucker et al., 2005), but LN-mediated mechanisms leading to enhanced growth are not well defined. We hypothesize that these mechanisms likely represent a complex interplay between enhancement of permissive cues and downregulation of those that are inhibitory. This study investigated potential interactions between the LN and NGF-mediated signaling pathways in PC12 cells and primary neurons. Elucidating neuronal growth or regeneration programs in a neuronal model, both in terms of neurotrophin responsiveness and effects of permissive growth environments is likely to provide insights into ways of enhancing CNS repair.

## **4.2 Materials and Methods**

### **4.2.1 Cell Culture and Differentiation**

The experimental model consisted of wild-type rat pheochromocytoma (PC12) cells, and a mutated PC12 derivative cell line (gifts from Dr. David Kaplan, Hospital for Sick Children, Toronto, ON). PC12nnr5 cells were derived by mutating parental PC12 cells using ethylmethanesulfonate, as described by Green et al. (1986).

Cell lines were maintained on rat-tail collagen-coated tissue-culture flasks in RPMI 1640 medium (Invitrogen, Burlington ON) supplemented with 10% horse serum (Invitrogen), 5% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin/glutamine solution (PSG; Invitrogen). Cells were incubated at 37 °C in 5% CO<sub>2</sub>, and cultured to 80% confluence prior to trypsinization for subculturing and differentiation. Cells were

differentiated by exposure to NGF (100 ng/ml) for 2 days in low serum conditions to prime, followed by exposure to NGF (100 ng/ml) for 5 days in the absence of serum.

#### **4.2.2 Primary Cell Culture**

Hippocampal cultures were prepared from postnatal day 0-2 (P0-2) Sprague-Dawley rat pups as previously described (Jiang et al., 2005). Briefly, the hippocampi were dissected and placed into ice-cold dissection buffer (Hank's Balances Salt Solution (HBSS; Invitrogen) supplemented with 1 mM HEPES buffer (Invitrogen) and 1% penicillin/streptomycin solution). The meninges were removed and tissue was minced. After washing in HBSS, tissue was trypsinized with 0.25% trypsin-EDTA for 15 minutes at 37 °C. Trypsinization was inhibited by the addition of fetal calf serum. Tissue was then washed with plating medium (EMEM supplemented with 10 mM sodium bicarbonate, 20 mM potassium chloride, 1 mM HEPES, 10% FCS and 1% PSG) and manually triturated with a flame-polished pipette 35 times to dissociate the cells. After washing and pelleting, cells were resuspended to a seeding density of  $0.25 \times 10^6$  cells/ml and plated onto PL-coated (40 µg/ml) 12-well tissue culture plates or 16 well chamber slides and incubated at 37 °C in 5% CO<sub>2</sub>. Between 2 and 4 hours following plating, media was removed and replaced with neurobasal media containing 2% B27 supplement, 2 mM HEPES and 1% PSG. On day in vitro 3 (3DIV), and every third day thereafter, half of the media was replaced and altered to include 20 µM cytosine arabinoside to discourage proliferation of the non-neuronal constituents.

### **4.2.3 Immunocytochemistry**

For immunocytochemical studies, cells were trypsinized and subcultured serum-free on Poly-D-Lysine (PL)- or Laminin (LN)-coated 16-well chamber slides for 24 hours in the presence or absence of NGF (50 ng/mL), prior to fixation in 4% paraformaldehyde for 15 minutes. Cells were then permeabilized with 0.1% Triton-X-100 and blocked with 10% normal goat serum in PBS for 1 hour at room temperature. Cells were incubated with either a monoclonal antibody directed against total tubulin (Sigma), TuJ1 (Chemicon, Temecula, CA) or the combination of a mouse monoclonal antibody directed against p75NTR (MC192, Oncogene) and a rabbit monoclonal antibody directed against PTEN (Cell Signaling) for 16 hours at 4° C. This was followed by incubation with Cy2- and Cy5-tagged secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) for 1 hour at room temperature. Finally slides were coverslipped with glycerol, and the receptor expression patterns were visualized using confocal laser scanning microscopy.

### **4.2.4 Growth Quantification**

All measurements of growth were obtained using images of PC12 cells immunostained for total tubulin (Sigma), or hippocampal neurons immunostained for neuronal marker, TuJ1 (Millipore). Individual cell tracings were performed using NIH Image J software. Only neurons for which we were able to unambiguously identify the associated neurites were chosen for tracing analysis. Neurite length is a measure of the longest neurite extending from each neuron (n>250 cells per condition). Data were



imported into a graphing and statistical analysis program (Prism 4, Graphpad Corp.) for further analysis.

#### **4.2.5 Western Blot Analysis**

For Western analyses, cells were harvested in the presence of sodium orthovanadate and subsequently subjected to lysis (10% glycerol, 1% NP-40, sodium vanadate, sodium fluoride, magnesium chloride, octyl- $\beta$ -thioglucopyranoside (Sigma), and 1 protease inhibitor cocktail tablet (Roche Scientific, Laval, QC) overnight at 4° C prior to centrifugation (10,000 rpm, 5 minutes). A BCA protein assay (Pierce, Rockford IL) was used to determine protein concentration and equivalent amounts of protein (50  $\mu$ g) were electrophoresed on 8% SDS-polyacrylamide gels. Protein was subsequently transferred to nitrocellulose membranes that were then exposed to Ponceau red to ensure equal protein loading. After washing in TBS, blots were blocked in 3% non-fat dry milk for 1 hour at room temperature, and then incubated with a primary antibody overnight at 4° C. Antibodies used include: Anti-Actin (Sigma), Anti-phospho-Akt (S473, Cell Signaling), Anti-Akt (Cell Signaling), Anti-ERK-44/42 (Santa Cruz), Anti-phospho-ERK-44/42 (Upstate), Anti-phospho-GSK3 $\beta$  (Ser9, Cell Signaling), Anti-GSK3 $\beta$  (Cell Signaling), Anti-Integrin  $\beta$ -1 (Chemicon), Anti-p75NTR ECD (MC192; Oncogene), Anti-p75NTR ICD (Upstate), Anti-PTEN (Cell Signaling), Anti-Rho (Upstate), and Anti-TrkA (Upstate). A final incubation with HRP-conjugated secondary antibody (AP307P, AP308P; Chemicon,) for 1 hour at room temperature was followed by visualization using an ECL method. Each experiment was repeated three times, subjected to densitometry and normalized to actin to ensure equal protein for comparison. Statistical analysis was

performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego CA) with significance being determined using one-way ANOVA testing.

#### **4.2.6 Real-time RT-PCR**

For real time RT-PCR analysis, cells were subcultured on PL or LN-coated 12-well plates under serum-starved conditions for 24 hours in the presence or absence of NGF (50 ng/ml). RNA was isolated using Trizol reagent (Invitrogen) as per the manufacturer's instructions and was subsequently treated with DNase (Ambion; Austin TX) to remove traces of contaminating DNA. RNA (2 µg) was reverse transcribed to cDNA using MMLV reverse transcriptase (200 U for 30 minutes at 37°C; Invitrogen) prior to use as template for real-time PCR amplification using the following PCR primer pairs: p75NTR forward 5'-TGCATCTGAGCTGGTGTCTGTCTT, p75NTR reverse 5'-TGCGTACAATGCTCCTGGTCTCTT, PTEN forward 5'-ATTCGACTTAGACTTGACCT, PTEN reverse 5'-ACCAGTCCGTCCTTTC, 28S forward 5'-GACCAAGGAGTCTAACGC, 28S reverse 5'-GTACGCTCGTGCTCCA. PCR amplification was performed using the Roche LightCycler (Roche) and quantified using SYBR green I. p75NTR and PTEN mRNA expression levels were subsequently normalized using the housekeeping gene, 28S.

#### **4.2.7 Rho Activity Assay**

The activation of RhoA was assessed using a pull-down assay kit (Upstate, Charlottesville, VA), in accordance with the manufacturer's instructions. Briefly, cells were harvested and lysed. Rhotekin-bound beads were added to cleared lysate and rotated for 1 hour at 4°C prior to pelleting, washing, and boiling in Laemmli buffer with



DTT. Rhotekin-bound Rho proteins were detected by Western blot analysis using a polyclonal anti-Rho antibody (Upstate, 06-770). The total amount of RhoA in cell lysate was used as a control for the cross-comparison of Rho activity.

#### **4.2.8 Plasmids and Transfection**

pcDNA3.1-p75NTR-orange: Plasmid sequence encoding orange fluorescent protein was excised from the pRSET vector (a kind gift from Dr. Lois Mulligan, Queen's University, Kingston ON) using BamHI and HindIII restriction enzymes, and was inserted into the eukaryotic expression vector pcDNA3.1 (Invitrogen). Amplification of the full length rat p75NTR coding region was performed using forward primer 5'-CTCGAGATGAGGAGGGCAGGTGCTGC-3' and reverse primer 5'-GATATCTGCACTGGGGATGTGGCAGTGG-3' encoding XhoI and EcoRV restriction sites, respectively, which facilitated directional, in-frame insertion into the engineered pcDNA3.1-orange vector yielding p75NTR with a C-terminus orange fluorescent tag.

The pcDNA3.1-p75NTR (wild-type) plasmid and empty vector control were kind gifts from Dr. Donna Senger (University of Calgary).

pIRES-PTEN: Full length rat PTEN coding sequence was amplified from cDNA using PCR forward primer 5'-GAATTC CATGACAGCCATCATC and reverse primer 5'-GGATCCTCAGACTTTTGTAATTTGTG incorporating EcoRI and BamHI restriction enzyme sequences, respectively. The resulting PCR product was cloned into the TOPO TA cloning vector 2.1 (Invitrogen), subsequently excised using EcoRI and BamHI, and directionally inserted into the bi-cistronic GFP reporter vector, pIRES (Promega).



Ras constructs: wild-type, V12 (constitutively active) and N17 (dominant negative), were purchased from Clontech.

All plasmids were transfected into differentiated PC12 cells using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions.

#### **4.2.9 Small interfering RNA constructs and transfection**

PTEN siRNA constructs were purchased from Cell Signaling (PTEN Signal Silence siRNA). PC12 cells were transfected with 100 nM of PTEN siRNA according to manufacturer's protocols. The siRNA transfection efficiency was assessed to be >70% based on transfection of Alexa fluor-labelled negative control siRNA (scrambled; target sequence AATTCTCCGAACGTGTCACGT; Qiagen, Mississauga ON). 48 hours post-transfection, cells were transferred onto PL or LN coated tissue culture plates for protein analysis, or 16-well chamber slides for growth analysis, after an additional 24 hours.

### **4.3 Results**

#### **4.3.1 Laminin enhances neurite regeneration via activation of early signaling intermediates essential to growth.**

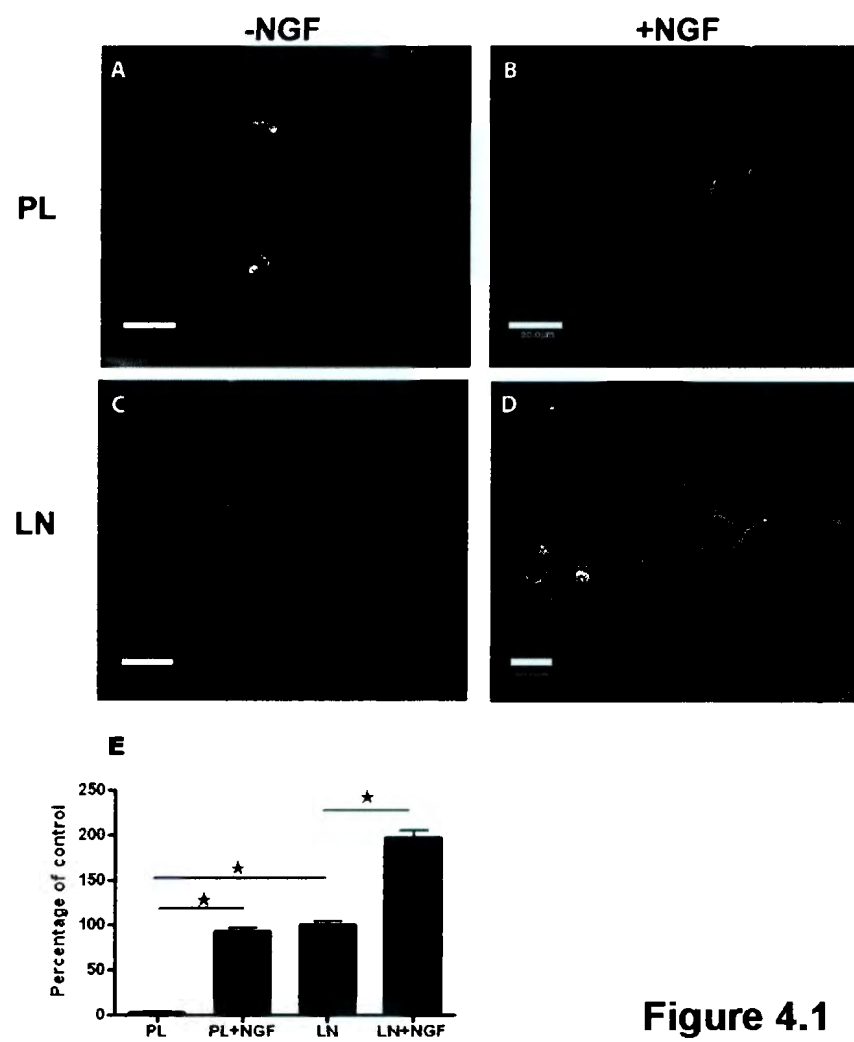
Our initial investigations into the potential mechanisms underlying the synergistic effects of NGF and LN on neurite outgrowth response were conducted using NGF-differentiated PC12 cells. As PC12 cells differentiate, they extend an extensive network of neurites (Greene and Tischler, 1976). After scraping the cells from the tissue culture surface and mechanically triturating with a flame-polished Pasteur pipette to remove neurite outgrowth, we then investigated regeneration of these neuritic processes under

various conditions. Cells plated on PL in the absence of additional growth factors showed very little regenerative response (Fig. 4.1A). While NGF (50 ng/ml; Fig 4.1B) or a LN substrate (Fig 4.1C) each independently elicited a growth response, it was the combination of NGF and LN (Fig 4.1D) that elicited the most robust growth response, in terms of length. Fig 4.1E presents the quantitation of neurite growth as described in Materials and Methods.

While effects of LN + NGF-initiated growth can be assessed at 24 hours post-plating, the initiating signals often occur much earlier. In fact, phosphorylation events leading to neurite outgrowth often occur within minutes of stimulation, and can be transient in nature (Tucker et al., 2006; Tucker et al., 2008). The phosphorylation of extracellular signal related kinase (ERK1/2) is essential for neurite outgrowth in PC12 cells (Fukuda et al., 1995; Pang et al., 1995; Xiao and Liu, 2003). Phosphorylation of ERK is a component of both Trk and integrin signaling cascades (Yee et al., 2008). To assess the activation of this early signaling intermediate, cells were plated on PL under serum-starved conditions for 24 hours prior to stimulation with either soluble NGF (50 ng/ml), soluble LN (25  $\mu$ g/ml) or both for time periods of 10 minutes, 30 minutes or 1 hour. Samples were then collected and protein prepared as described. Western blot assessment of phospho-ERK at each timepoint following stimulation showed significantly enhanced phosphorylation follows co-stimulation relative to phospho-ERK activation by either stimulus alone (Fig. 4.2 A). Interestingly, the phosphorylation of ERK by integrin activation was both delayed and shorter-lived relative to NGF signaling. To demonstrate the effects of ERK signaling on neurite outgrowth in PC12 cells, we manipulated the phosphorylation of ERK using mutated Ras constructs and examined the resultant effects

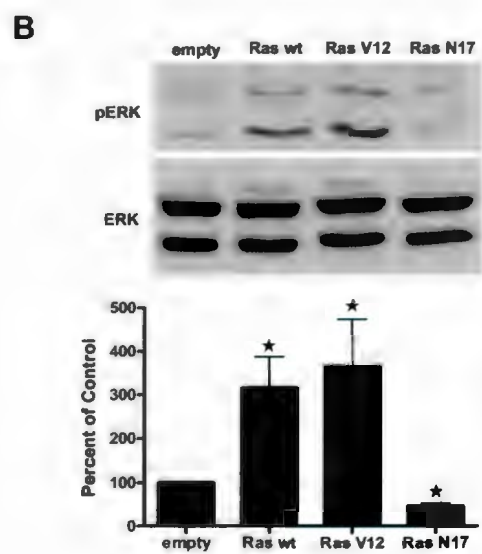
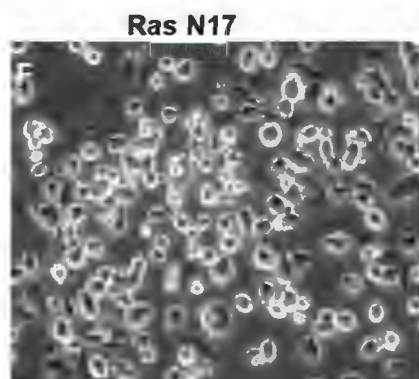
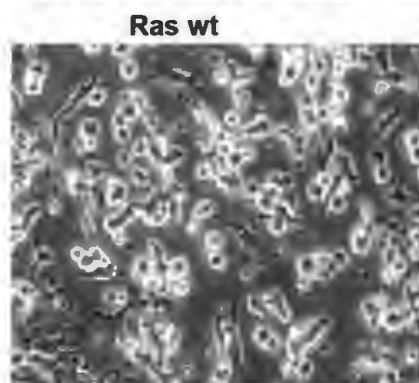
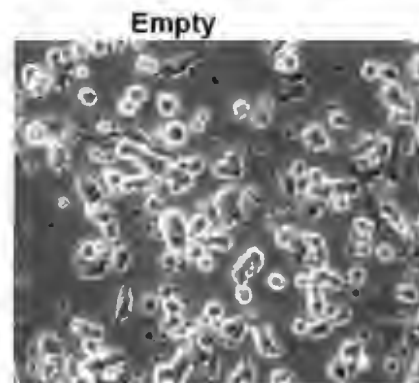
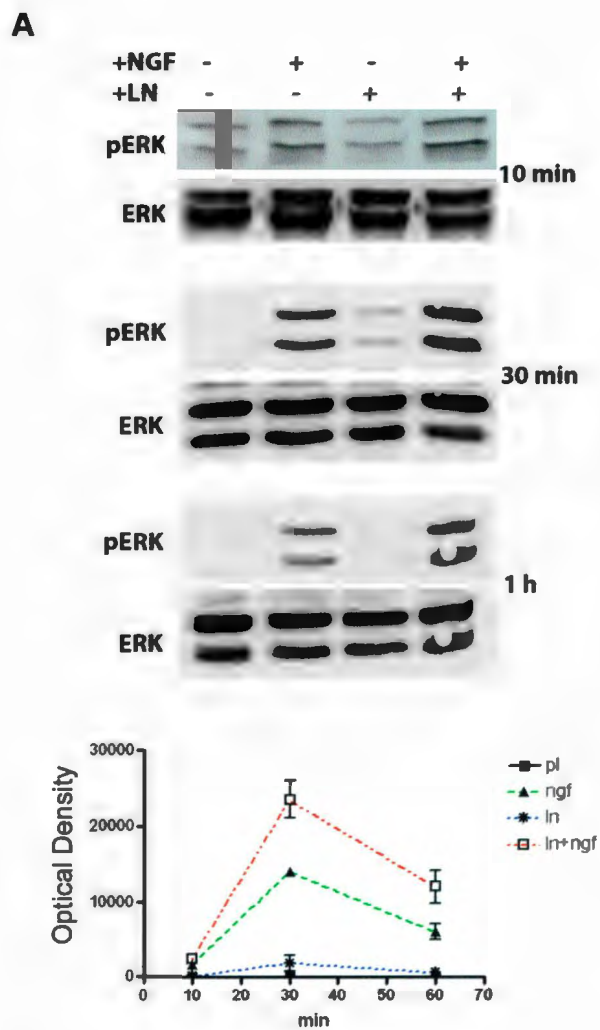
**Figure 4.1: Neurite regeneration is enhanced on a LN substrate.** A-D: Confocal images of differentiated PC12 cells immunostained for total tubulin demonstrate the enhancement of neurite outgrowth on a permissive LN substrate relative to the neutral PL substrate, both in the presence and absence of NGF. E: Graphical representation of neurite length measurements obtained using Image J, and presented as percent of LN control. Scale bar -20  $\mu\text{m}$ , \* $p < 0.001$  ANOVA.





**Figure 4.1**

**Figure 4.2: Phosphorylation of ERK is enhanced in the presence of NGF and LN co-stimulation.** (A) Differentiated PC12 cells were plated on PL overnight in serum-starved conditions in the absence of NGF prior to stimulation with soluble NGF, soluble LN or both for 10 minutes, 30 minutes or 1 hour. Western blotting shows an increase in the phosphorylation of early signaling intermediate ERK in the presence of NGF, which is significantly enhanced with LN co-stimulation at each timepoint tested. (B) Manipulation of ERK phosphorylation was accomplished using Ras V12 to enhance phosphorylation of ERK and Ras N17 to prevent it. (C-F). Enhanced phosphorylation of ERK was associated with enhanced neurite outgrowth. Values expressed represent the mean protein expression, relative to ERK, of 3 experiments  $\pm$  SEM.



**Figure 4.2**



on neurite outgrowth. Wild-type Ras, constitutively active Ras (V12) or dominant negative Ras (N17) were transfected into PC12 cells plated on PL. The introduction of wild-type Ras or Ras V12 increased the phosphorylation of ERK and elicited neurite outgrowth. Conversely, Ras N17 decreased the phosphorylation of ERK relative to empty vector control, and was not associated with a neurite outgrowth response (Fig. 4.2 B-F).

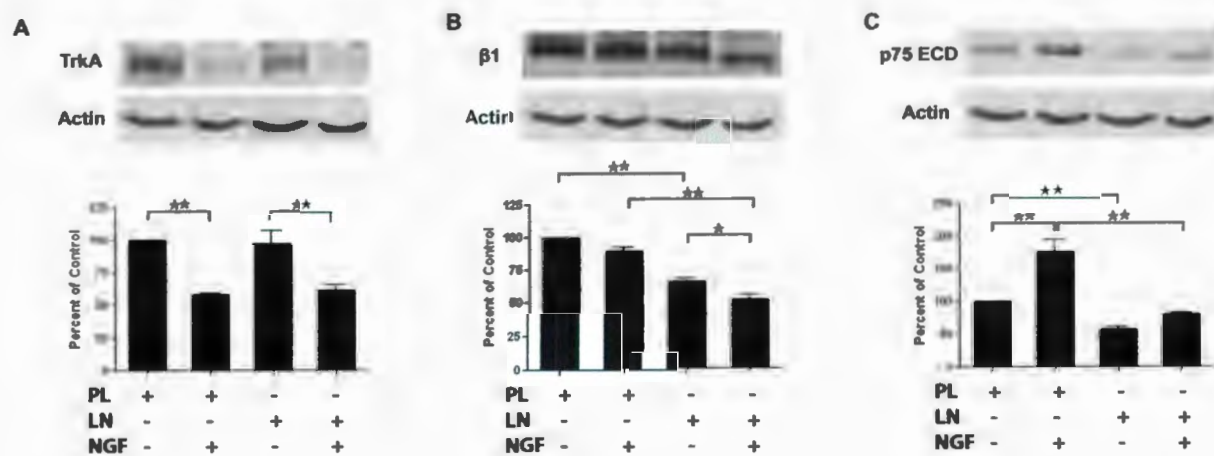
#### **4.3.2 Laminin decreases p75NTR expression by a mechanism involving both transcriptional downregulation and cleavage.**

We next investigated the effects of NGF and LN exposure on the expression of the relevant surface receptors. Cells subcultured on PL versus LN in the presence or absence of NGF for 24 hours were sampled and protein assessed for TrkA, integrin  $\beta 1$  and p75NTR expression levels. Results showed that NGF downregulated TrkA expression (Fig. 4.3A) and LN downregulated its receptor, integrin  $\beta 1$  (Fig. 4.3B). Suppression of these cell surface receptors was ligand-specific, as NGF stimulation alone did not modulate integrin  $\beta 1$  while LN exposure did not affect expression of TrkA. NGF upregulated p75NTR (Fig. 4.3C) as previously reported (Rankin et al., 2005), but perhaps most interestingly, the LN substrate resulted in decreased detection of p75NTR (Fig. 4.3C).

Since the antibody used to detect p75NTR (clone MC192) was specific for the extracellular domain of p75NTR, and p75NTR is known to undergo ectodomain shedding and secretase-mediated cleavage (Kanning et al., 2003), we further investigated this

**Figure 4.3: LN decreases the detection of p75NTR extracellular domain.**

Differentiated PC12 cells were subcultured on PL versus LN substrate in the presence or absence of NGF for 24 hours. Western blotting assessment of TrkA expression reveals a decrease following NGF exposure, but LN had no significant effect (A). Integrin  $\beta 1$  was significantly reduced following exposure to the LN substrate, but NGF alone had no effect on protein expression (B). In contrast, NGF upregulates p75NTR expression, while LN significantly reduced the detectability of p75NTR protein, as assessed using an antibody directed against the ECD of the receptor (C). Values expressed represent the mean protein expression, relative to actin, of 3 experiments  $\pm$  s.e.m. \* $p < 0.05$ , \*\* $p < 0.001$  (ANOVA).



**Figure 4.3**



apparent downregulation. Differentiated PC12 cells were subcultured on PL versus LN substrates in the presence or absence of NGF for 24 hours. Western blot detection using an antibody specific for the intracellular domain of p75NTR also showed a downregulation in response to the LN substrate (Fig. 4.4A). Assessment of mRNA encoding p75NTR was performed using quantitative real-time RT-PCR, and confirmed that the downregulation of p75NTR also occurred on the transcriptional level (Fig. 4.4B). However, cleavage of the receptor cannot be excluded since a minor amount of a 25 kDa low molecular weight cleavage fragment was identifiable by Western blot analysis using the intracellular domain-specific p75NTR antibody, and is enhanced on the LN substrate (Fig. 4.4C). This low level of cleaved p75NTR could only be detected upon overexposure of the Western blot, and is in low abundance relative to the full-length receptor (Fig 4.5).

#### **4.3.3 Overexpression of p75NTR prevents neurite extension in differentiated PC12 cells.**

To establish a direct link between p75NTR expression and the inhibition of neurite growth in these cells, we generated a full length p75NTR expression construct which incorporated a carboxy-terminal orange fluorescent tag (Fig. 4.6 A), and used it to compare the regenerative capacity of differentiated PC12 cells transfected with the p75NTR fusion construct versus cells expressing the control vector. Differentiated PC12 cells were transfected with pcDNA3.1-p75NTR-orange or pcDNA3.1-orange control vectors for 24 hours, scraped and triturated to remove all neurite outgrowth, and then replated on LN. After 36 hours exposure to the LN substrate, the cells were fixed and

**Figure 4.4: LN-mediated downregulation of p75NTR is dependent on reduced transcription and enhanced cleavage.** Differentiated PC12 cells were subcultured on PL versus LN substrates in the presence or absence of NGF for 24 hours. LN induces a significant downregulation of p75NTR intracellular domain protein (A), and mRNA (B) as assessed by real time RT-PCR. Concomitantly, LN is reported to induce matrix metalloproteinases, which can mediate p75NTR ectodomain shedding. In support of this paradigm, Western blotting reveals a LN-mediated increase in a smaller molecular weight p75NTR cleavage product (C), which is in low abundance relative to the full length receptor (see Suppl. Fig. 1). Values expressed represent the mean protein expression relative to actin or 28S, of 3 experiments  $\pm$  s.e.m. \* $p < 0.05$ , \*\* $p < 0.001$  (ANOVA).

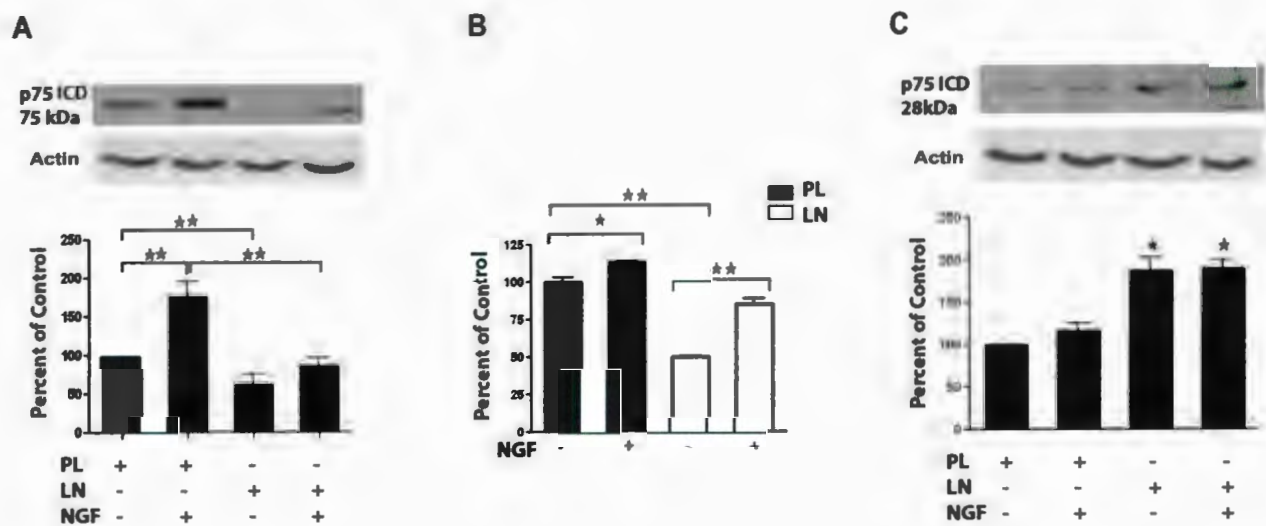
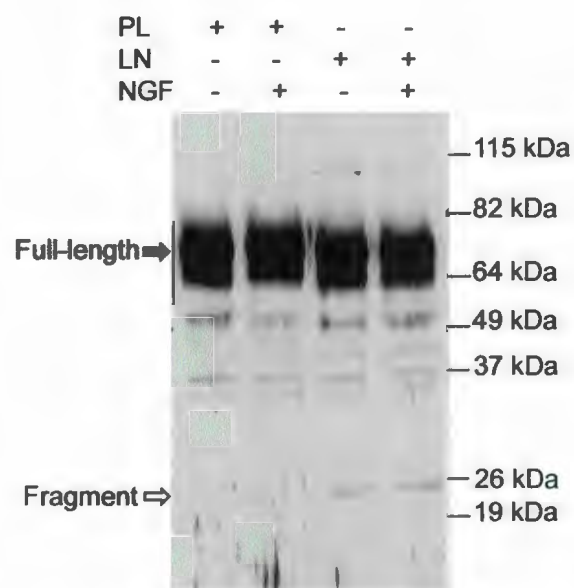


Figure 4.4



**Figure 4.5: Low molecular weight p75NTR fragments are in low abundance relative to the full-length receptor.** As illustrated in Fig. 4C, Western blotting reveals a LN-mediated increase in a smaller molecular weight p75NTR cleavage product. Here we show an image of the full blot to demonstrate that the cleaved band was only detectable after overexposure of the Western blot, and is thus in low abundance. This illustrates that cleavage may only be playing a very small role in the cellular decrease in p75NTR, and that transcriptional downregulation shown in Fig. 4B may be playing a larger role.



**Figure 4.5**

**Figure 4.6: p75NTR overexpression impairs neurite extension.**

A cDNA construct consisting of p75NTR with a C-terminus orange fluorescent tag was cloned into expression vector pcDNA3.1 as described, and can be detected as a fusion protein by Western blotting using a p75NTR ICD antibody (A). Differentiated PC12 cells were transfected with either empty control vector or pcDNA3.1-p75NTR-orange fusion vector or pcDNA3.1-p75NTR wild type prior to transfer onto a LN-coated substrate. After 36 hours, cells expressing the empty vector had normal neurite extension in response to LN exposure (B, D-F), but this growth was effectively stunted in cells expressing the p75NTR-orange fusion protein (C), or the wild-type p75NTR (G-I). Anti-p75NTR ICD was used to detect wild type p75NTR, and visualization was achieved using Cy3-tagged secondary (red). These cells were co-stained with total tubulin (green) to visualize neurite outgrowth. Arrow indicates cell overexpressing p75NTR wild-type construct.



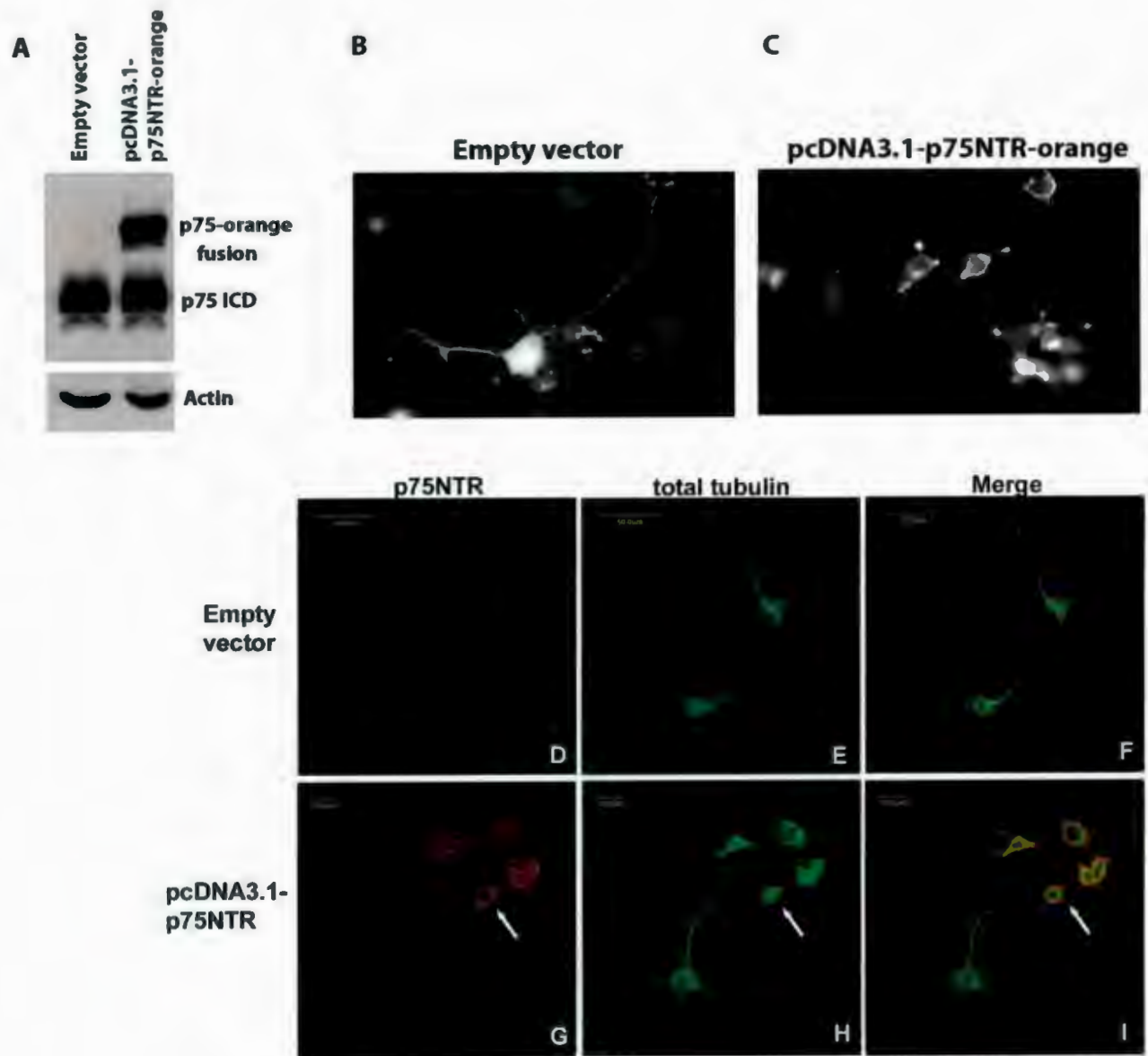


Figure 4.6

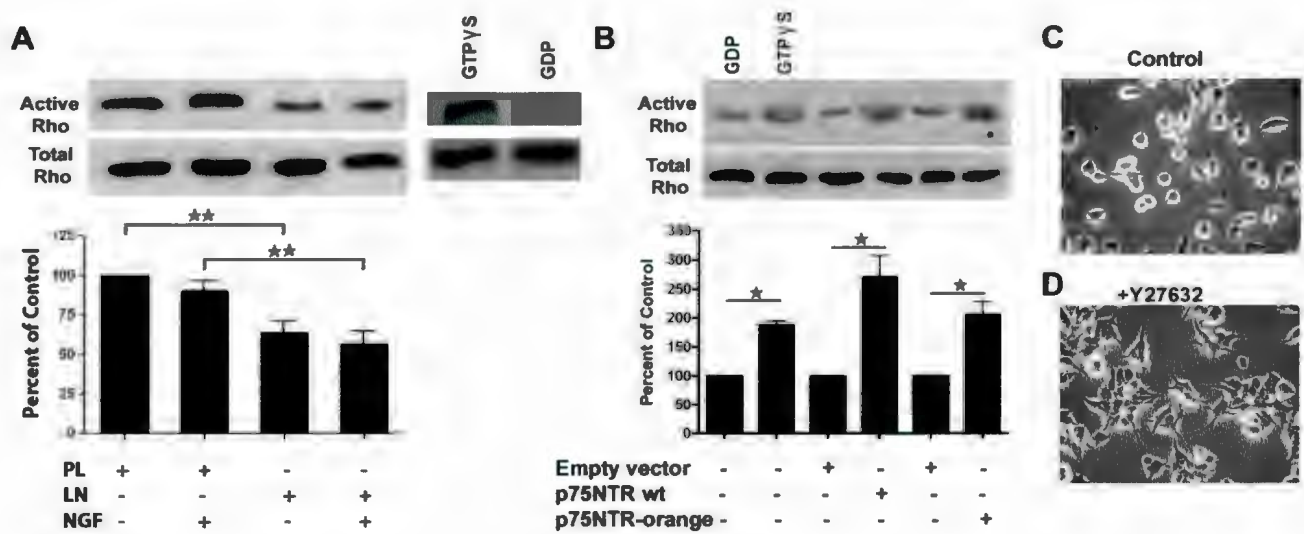
imaged to assess growth of the transfected cells. While those cells expressing the empty vector responded to the LN substrate by extending long neuritic processes, the overexpression of p75NTR largely stunted neurite extension, or prevented initiation of neurite growth (Fig. 4.6 B,C). Because of the possibility that by placing the tag on the C-terminus, we have potentially interfered with the function of p75NTR, these experiments were repeated using a wild-type p75NTR construct (Esposito et al., 2001). The results show that there were no obvious differences between the two constructs in the parameters being assessed. Relative to both the untransfected cells present in the same frame and the empty vector transfected cells (Fig 4.6D-F), increased levels of p75NTR were associated with short stunted neurites (Fig 4.6G-I).

#### **4.3.4 Laminin induces long-term depression of Rho activity.**

Since p75NTR constitutively activates Rho GTPase, a known inhibitor of neurite outgrowth (Yamashita et al., 1999), and p75NTR siRNA has been shown to decrease Rho activity, thus promoting neurite outgrowth (Ahmed et al., 2005), we assessed Rho activity as a physiological consequence of downregulating p75NTR expression. Differentiated PC12 cells were subcultured on PL versus LN substrates in the presence or absence of NGF for 24 hours. Active Rho was analyzed in protein samples using a Rho activity assay kit as described in Materials and Methods. In support of our initial experiments which demonstrated significant reductions of p75NTR upon exposure to a LN substrate, we detected decreased Rho activity on a LN substrate in both the presence and absence of NGF (Fig. 4.7 A) suggesting that LN-mediated downregulation of p75NTR may be of physiological relevance. The lack of effect on Rho activity observed in the presence of

**Figure 4.7: LN induces long-term depression of Rho activity.** Differentiated PC12 cells were subcultured on PL versus LN substrates in the presence or absence of NGF for 24 hours. Active Rho was assessed in protein samples using a Rho activation assay kit (Upstate), utilizing Rho-Binding domain conjugated beads to capture and precipitate active Rho (A). Overexpression of p75NTR-wild type or p75NTR-orange constructs resulted in increased activation of Rho (B). GTP $\gamma$ S loaded samples served as the positive control, while GDP loaded samples served as the negative control. Values expressed represent the mean of 3 experiments  $\pm$  SEM \*\* $p < 0.001$  (ANOVA). Inhibition of Rho effector, Rock, using Y27632 (10 $\mu$ M) resulted in enhanced neurite outgrowth (C-D).





**Figure 4.7**

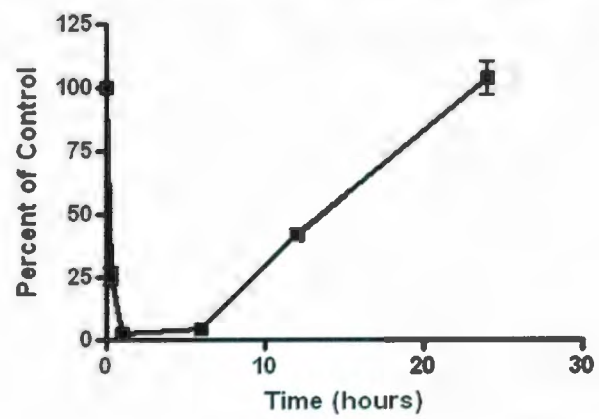
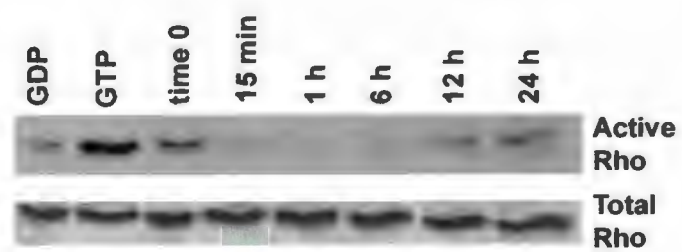
NGF can be explained by the 24 hour timepoint. NGF inactivation of Rho occurs rapidly and returns to baseline by 24 hours (Moissoglu and Schwartz, 2006) (Fig. 4.8). To confirm a role for p75NTR in the modulation of Rho activity, we overexpressed empty control vectors versus both the wild-type p75NTR and the p75NTR-orange constructs and performed the assay to quantify activated Rho. Both the tagged and untagged p75NTR constructs resulted in enhanced Rho activity (Fig. 4.7 B). To demonstrate the impact of Rho signaling on neurite outgrowth in differentiated PC12 cells, we used the pharmacological inhibitor Y27632 (10 $\mu$ M) to inhibit the activity of the downstream Rho effector, Rho associated kinase (Rock). The introduction of the Rock inhibitor promoted the extension of neurite outgrowth in differentiated PC12 cells plated on PL (Fig. 4.7 C-D). These results support the contention that sustained p75NTR-mediated activation of Rho (as observed with the enhanced expression of p75NTR) is key in the inhibition of neurite growth.

#### **4.3.5 LN-mediated downregulation of p75NTR is associated with an upregulation of PTEN.**

We attempted to investigate the mechanism between integrin activation and modulation of p75NTR expression. A previously reported cell line stably overexpressing PTEN was characterized to express very low levels of p75NTR (Musatov et al., 2004). Thus, we investigated a potential role for PTEN in the regulation of p75NTR in the present system. Differentiated PC12 cells were plated on PL versus LN substrates in the presence or absence of NGF for 24 hours prior to protein collection for subsequent Western blot analysis. As demonstrated in Figure 4.9A, LN significantly upregulated

**Figure 4.8: NGF induces a transient suppression of Rho activity which returns to baseline by 24 hours.** Differentiated PC12 cells were plated on PL for 24 hours prior to stimulation with NGF (50  $\mu\text{g/ml}$ ), collected at the indicated timepoints and used in a pulldown assay for the detection of active Rho. GDP-loaded samples served as negative control, while GTP $\gamma$ S-loaded samples served as a positive control. The total amount of RhoA in cell lysates was used as a control for the cross-comparison of Rho activity.





**Figure 4.8**

**Figure 4.9: LN upregulates PTEN expression, which causes p75NTR**

**downregulation.** Differentiated PC12 cells were subcultured on PL versus LN substrates in the presence or absence of NGF for 24 hours. Western blotting reveals an upregulation of PTEN in cells plated on the LN substrate, while NGF has no effect on PTEN expression (A). Transient transfection of pIRES-PTEN resulted in successful PTEN overexpression (B) which correlated with a downregulation of p75NTR expression (C). Values expressed represent the mean protein expression, relative to actin (A) or MAPK (B) of 3 experiments  $\pm$  s.e.m.  $**p < 0.0001$ . Cells were immunostained and imaged using confocal microscopy to illustrate the relative intensity and localizations of p75NTR (green) and PTEN (red) (D-O). Scale bar -20  $\mu$ m.

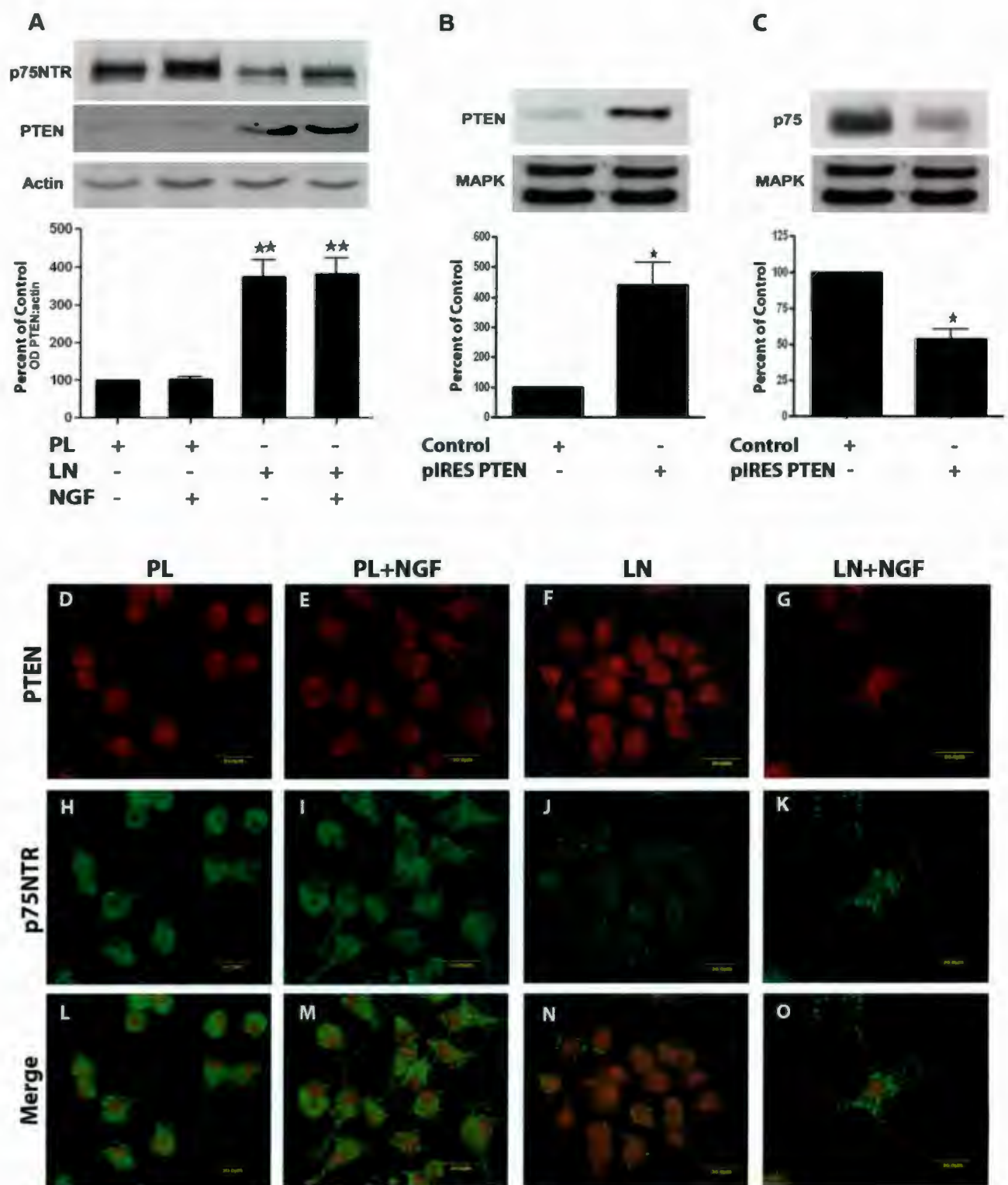


Figure 4.9



PTEN expression in differentiated PC12 cells in comparison with PL control cultures. A direct link between PTEN and p75NTR expression was established using a bicistronic pIRES-PTEN construct as described in Materials and Methods. The pIRES-PTEN or pIRES control constructs were introduced into differentiated PC12 cells using Lipofectamine 2000 reagent. Western blot analysis of protein lysates collected 24 hours post transfection showed that PTEN overexpression was associated with decreased levels of p75NTR (Fig 4.9 B-C). The reciprocal expression of p75NTR and PTEN upon exposure to a LN substrate was also confirmed by immunocytochemistry (Fig 4.9 D-O). To confirm that LN was inducing the upregulation of PTEN and downregulation of p75NTR via the integrin receptors, we utilized an inhibitor of integrin  $\beta 1$ , RGD peptide (Ruoslahti, 1996; Wildering et al., 1998). PC12 cells were pre-incubated in RGD (20 $\mu$ M) for 2 h prior to plating on PL or LN substrates. Cells that were exposed to the RGD prior to LN exposure did not upregulate PTEN or downregulate p75NTR to the same degree as those not inhibited by RGD peptide (Fig 4.10), thereby confirming that these LN signals are mediated by integrin receptors.

#### **4.3.6 Silencing of PTEN increases p75NTR expression and impairs neurite regeneration on LN.**

To further establish that PTEN may regulate the expression of p75NTR, we used siRNA constructs to suppress PTEN protein expression. 48 hours following transfection with the siRNA constructs, differentiated PC12 cells were transferred to PL or LN coated substrates for a further 24 hours prior to protein collection for Western blotting, or analysis of growth. Suppression of PTEN protein expression (Fig 4.11A) was associated

**Figure 4.10: Inhibition of integrin  $\beta$ 1 signaling prevents LN-induced upregulation of PTEN and p75NTR.** PC12 cells were pre-incubated with 20  $\mu$ M RGD peptide for 2 h prior to plating on PL or LN substrates. Western blot analyses show that inhibition of integrin  $\beta$ 1 blocks the upregulation of PTEN (A) and the downregulation of p75NTR (B) upon exposure to LN. Values expressed represent the mean protein expression, relative to actin, of 3 experiments  $\pm$  SEM. \* $p < 0.01$ , (ANOVA).

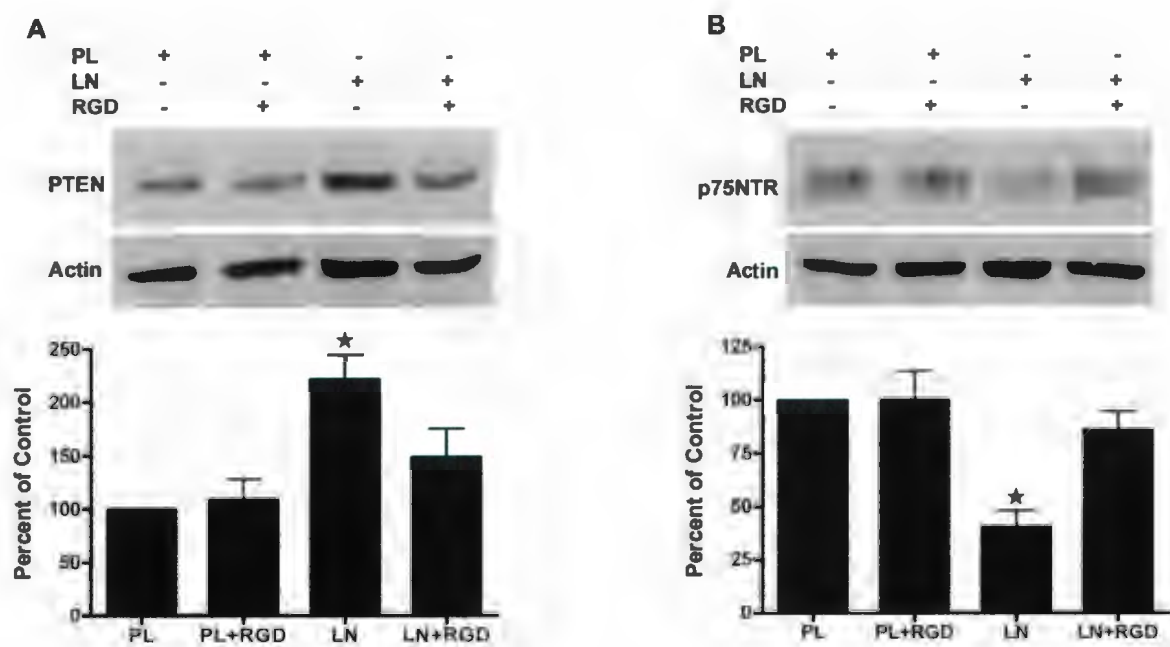
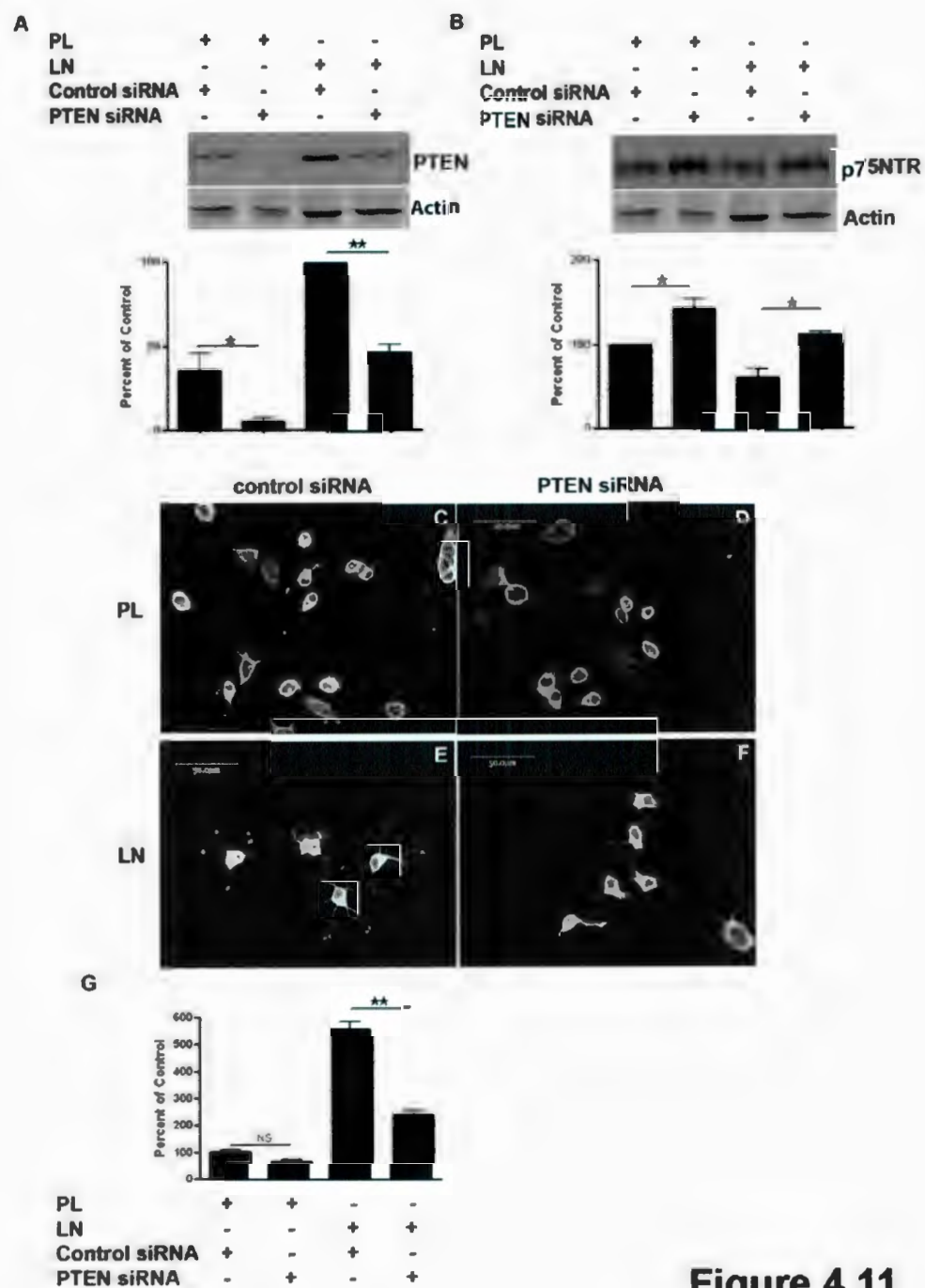


Figure 4.10



**Figure 4.11: Suppression of PTEN increases p75NTR expression and impairs neurite regeneration.** Differentiated PC12 cells were transfected with either control siRNA or PTEN siRNA constructs 48 hours prior to subculturing on PL or LN-coated substrates for an additional 24 hours. Western blot analyses reveal that specific knockdown of PTEN protein (A) is associated with an increase in p75NTR expression (B). Analysis of growth response demonstrates no noticeable impact of PTEN suppression associated with the PL substrate (C,D), but significant growth impairment of cells lacking PTEN was evident in response to LN substrate relative to control (E,F). This observation was confirmed by measuring neurite length (G). Values expressed represent the mean protein expression, relative to actin, of 3 experiments  $\pm$  s.e.m. \* $p < 0.05$ , \*\* $p < 0.001$  (ANOVA). Scale bar -50  $\mu\text{m}$ .



**Figure 4.11**

with an increase in p75NTR expression on both PL and LN substrates relative to a control siRNA (Fig 4.11B). Analysis of growth revealed that cells plated on PL showed very little regenerative response regardless of PTEN protein expression (Fig 4.11C-D). In contrast, the LN substrate elicited a growth response that was significantly impaired following PTEN knockdown (Fig 4.11E-F). Fig 4.11G presents quantitation of the neurite growth response.

#### **4.3.7 LN regulation of p75NTR expression does not require TrkA activity.**

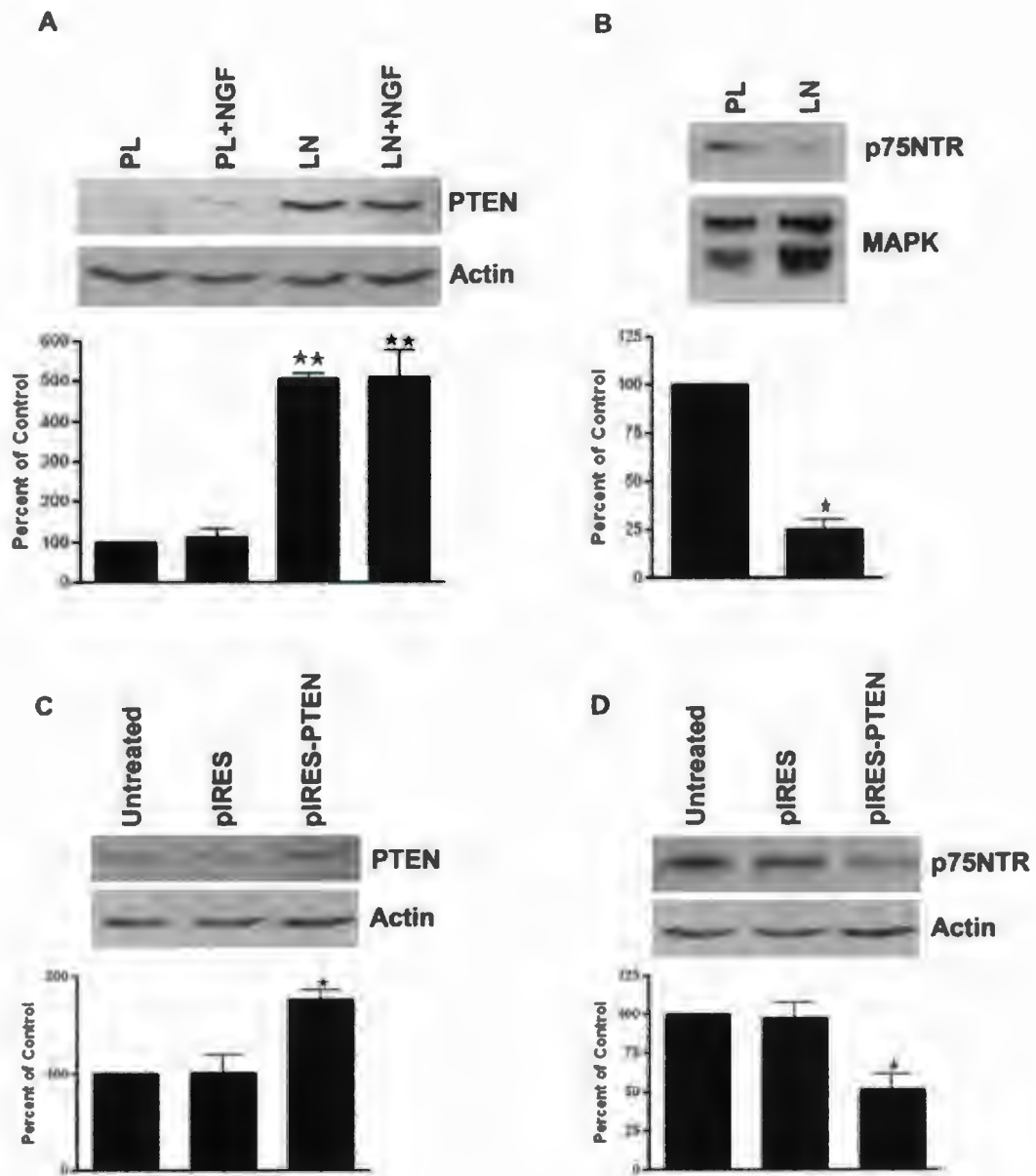
Because the cell line previously reported to overexpress PTEN displayed low levels of TrkA, as well as low levels of p75NTR (Musatov et al., 2004), and TrkA has been shown to modulate p75NTR expression (Rankin et al., 2005; Rankin et al., 2008), the possibility existed that TrkA contributed to the observed levels of p75NTR. To explore this possibility we utilized a mutated cell line that does not express TrkA, PC12nnr5. The PC12nnr5 cells were plated on PL versus LN in the presence or absence of NGF for 24 hours. Western blotting showed that LN upregulated PTEN expression (Fig. 4.12A) and downregulated p75NTR (Fig. 4.12B) in PC12nnr5. Furthermore, PTEN overexpression in PC12nnr5 cells via transient transfection also decreased p75NTR expression relative to untreated and empty vector controls (Fig. 4.12C,D). Thus the presence of TrkA was not required for LN-mediated downregulation of p75NTR.

#### **4.3.8 LN-induced upregulation of PTEN impacts the PI3K signaling cascade**

The primary role of PTEN is to negatively regulate the phosphatidylinositol-3-kinase (PI3K) signaling cascade, by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P<sub>3</sub>) into phosphatidylinositol-4,5-bisphosphate (PI(4,5)P<sub>2</sub>), and



**Figure 4.12: Regulation of p75NTR expression by LN is independent of TrkA activity.** PC12nnr5 cells, which lack endogenous TrkA, were plated on PL versus LN in the presence or absence of NGF for 24 hours. Western blotting reveals that LN upregulates PTEN expression (A), and downregulates p75NTR (B) in these cells. Furthermore, PTEN overexpression via transient transfection (C) also decreases p75NTR expression in these cells relative to untreated and empty vector controls (D). Values expressed represent the mean protein expression, relative to actin or MAPK, of 3 experiments +/- s.e.m. \* $p < 0.01$ , \*\* $p < 0.001$  (ANOVA).



**Figure 4.12**

thereby inhibiting the phosphorylation of Akt, and GSK resulting in downstream effects that could impact both survival and growth (Downes et al., 2001). In this regard, PTEN overexpression has previously been reported to decrease the phosphorylation of GSK (Beckner et al., 2005). In order to assess the impact of the LN-induced upregulation of PTEN on the PI3-K signaling cascade, differentiated PC12 cells were subcultured on PL versus LN for 24 hours, prior to stimulation with soluble NGF (50 ng/ml) for 10 minutes prior to protein collection. Subsequent Western blotting revealed that, while exposure to a LN substrate did not change the total amount of Akt that is phosphorylated in response to a short-term NGF stimulation, it did decrease the proportion of phosphorylated Akt as LN exposure appeared to upregulate Akt (Fig 4.13A). Similarly, there is a significant decrease in the proportion of available GSK3 that was phosphorylated in cells plated on the LN substrate prior to stimulation (Fig. 4.13B). Thus, the LN-induced upregulation of PTEN has an impact on the PI3K signaling cascade, but the influence on growth and survival does not appear to be detrimental suggesting either compensatory mechanisms employed by the cell or the existence of parallel signaling pathways initiated by the ECM and neurotrophin stimulation.

#### **4.3.9 A LN-mediated PTEN-p75NTR signaling cascade may promote neurite outgrowth in neonatal hippocampal neurons.**

While the LN-mediated upregulation of PTEN and subsequent downregulation of p75NTR results in enhanced regenerative neurite outgrowth in NGF-differentiated PC12 cells, a sympathetic neuron model, we sought to determine its physiological relevance in the central nervous system, specifically hippocampal neurons. Hippocampi were



**Figure 4.13: LN-mediated upregulation of PTEN affects the PI3K signaling pathway.**

In order to determine whether the LN-mediated increase in PTEN expression was affecting the PI3K signaling cascade, differentiated PC12 cells were plated on PL versus LN for 24 hours prior to stimulation with NGF (50 ng/ml) for 10 minutes. Subsequent Western blotting of protein lysates reveals that NGF stimulation phosphorylates Akt regardless of substrate, but LN appears to upregulate Akt, resulting in a decrease in the proportion of available Akt being activated (A). The LN substrate also decreases the proportion of GSK phosphorylated (B). Values expressed represent the mean ratio of optical density (OD) of the phosphorylated protein relative to the total protein of 3 experiments  $\pm$  SEM. \* $p < 0.01$ .

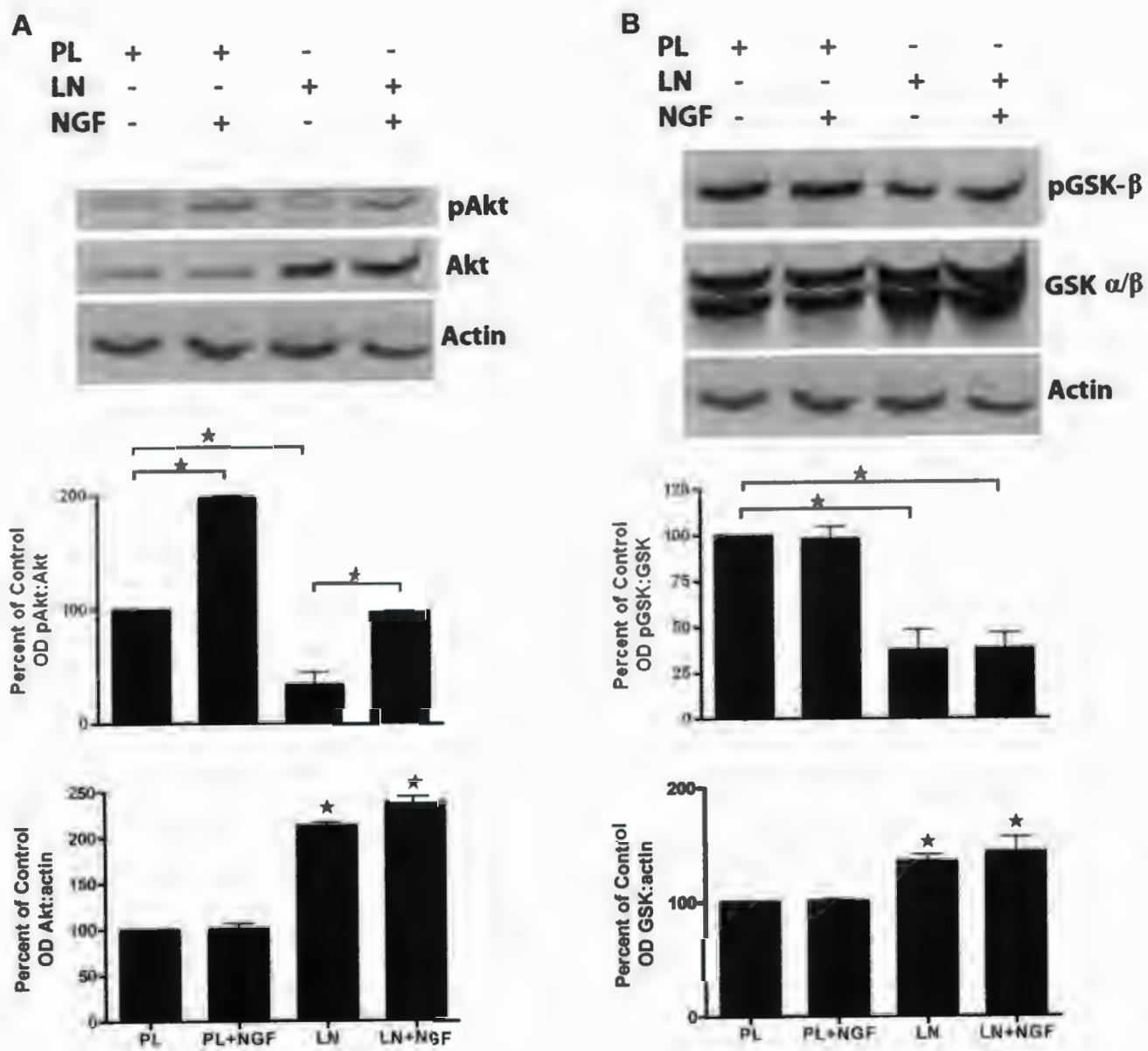


Figure 4.13

dissected from postnatal day 0-2 rat pups and cultured on PL versus LN substrate as described in Materials and Methods. Western blotting showed that LN upregulated PTEN and downregulated p75NTR protein expression (Fig. 4.14A,B). Using real time RT-PCR to quantify the mRNA, we confirmed a transcriptional upregulation of PTEN (Fig. 4.14C) and a transcriptional downregulation of p75NTR (Fig. 4.14D) in response to the LN substrate. Finally, we confirm that LN upregulates PTEN and downregulates p75NTR using immunocytochemistry (Fig. 4.14 E-J).

To assess the initial growth responses of these neurons to the different substrates, neurons were cultured on PL or LN coated chamber slides, fixed 24 hours post-plating, and stained for neuronal marker, TuJ1, to visualize the initial extension of processes from neurons, which were subsequently traced for length measurements as described in Materials and Methods. The LN substrate appears to be associated with an enhanced extension of process outgrowth relative to the PL substrate (Fig. 4.14 K,L), which is confirmed by the growth quantification (Fig. 4.14M). Thus, these results confirm that the LN-induced upregulation of PTEN and downregulation of p75NTR is consistent with a role in the growth of hippocampal neurons, as it is in PC12 cells.

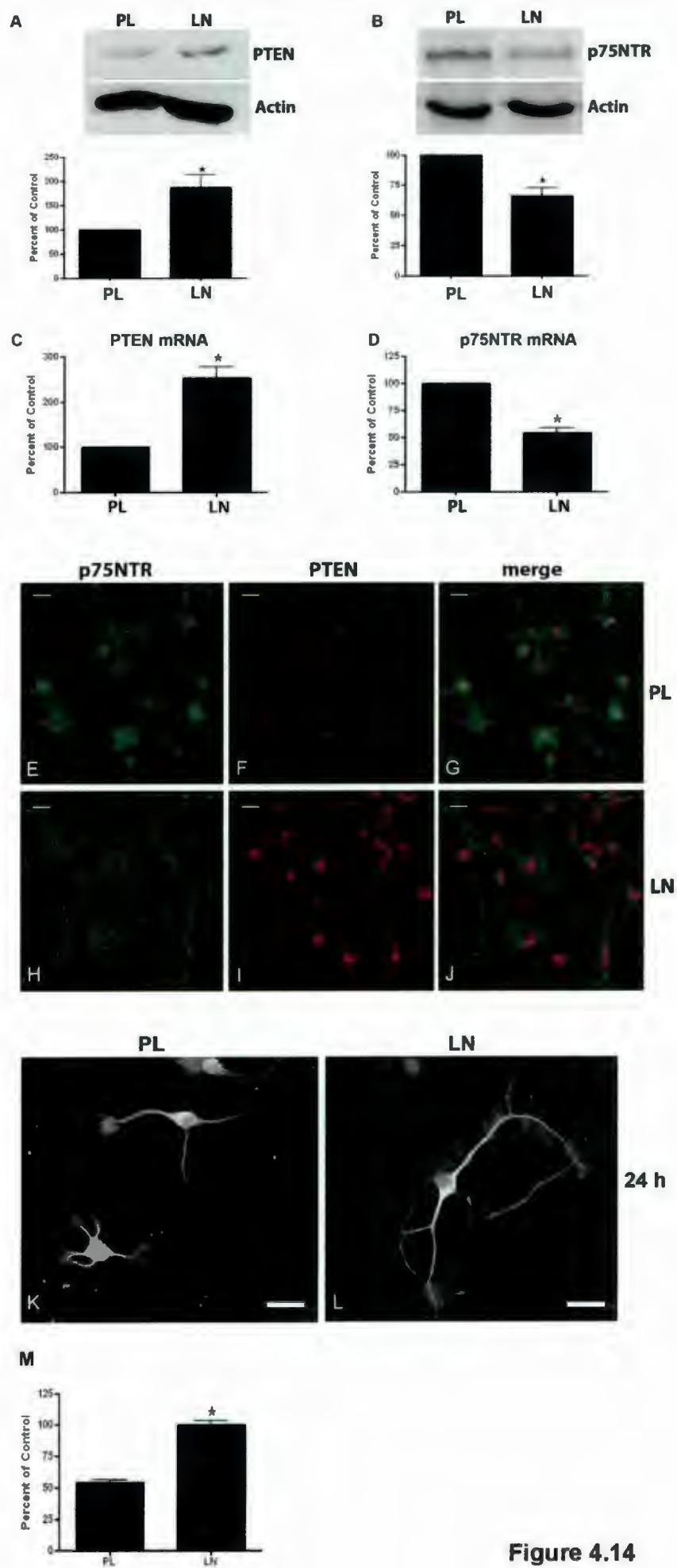
#### **4.4 Discussion**

This study used NGF-differentiated PC12 cells to explore a novel signaling pathway by which the ECM molecule, LN, enhances neurite outgrowth, by not only amplifying the phosphorylation of early signaling intermediates essential for growth, but also by diminishing surface expression of a potentially inhibitory receptor, p75NTR (Fig.



**Figure 4.14: Hippocampal neurons show evidence of a LN-mediated PTEN-p75<sup>NTR</sup> signaling cascade that enhances growth.**

To determine if the LN-mediated PTEN-p75<sup>NTR</sup> signaling cascade promotes growth in neonatal hippocampal neurons, hippocampi were dissected from P0-2 rat pups and cultured on PL versus LN substrates prior to harvesting for protein and RNA. Western blotting reveals that the LN substrate is associated with increased PTEN (A) and decreased p75<sup>NTR</sup> protein expression (B), and real-time RT-PCR confirms a transcriptional upregulation of PTEN mRNA (C) and downregulation of p75<sup>NTR</sup> (D). Neurons were immunostained and imaged using confocal microscopy to illustrate the relative intensity and localizations of p75<sup>NTR</sup> (green) and PTEN (red) (E-J). Growth was visualized following 24 hours in vitro by immunostaining with neuronal marker TuJ1. Neurite extension is enhanced on the LN substrate (L) relative to the PL substrate (K). This observation was confirmed by measuring neurite length (M). Values represent mean protein expression relative to actin, or mean mRNA expression normalized to 28S of 3 experiments +/- s.e.m. \* $p < 0.001$  (ANOVA). Scale bar -25  $\mu\text{m}$ .



**Figure 4.14**

4.15). We show that LN acts in an integrin-dependent manner to increase the expression of PTEN and decrease the expression of p75NTR. Using both exogenous expression and siRNA-mediated downregulation, our results demonstrate that PTEN is important in the LN-dependent enhancement of neurite growth. We also show that p75NTR plays a key role in inhibition of neurite growth in the absence of neurotrophins, likely via its effects on activation of Rho; overexpression of p75NTR results in decreased growth and sustained activation of Rho. We suggest that LN, via its actions on PTEN, results in the downregulation of p75NTR, and thus removal of its inhibitory influence.

NGF-differentiated PC12 cells share many properties with cultured sympathetic ganglion neurons, including the initiation of neurite outgrowth in response to NGF on certain substrates (Greene and Tischler, 1976). ECM components can also work in cooperation with NGF-induced signaling pathways to mediate significant neurite outgrowth in PC12 cells (Ishii et al., 2001; Vogelezang et al., 2001), and differentiated PC12 cells plated on a LN substrate can undergo NGF-independent neurite formation (Begovac and Shur, 1990; Glowacka et al., 1992), which we have confirmed in the present study (Fig. 4.1). The ligation of integrins by ECM components provides a multitude of contextual and signaling information to orchestrate a wide variety of biological processes, including proliferation, differentiation, migration, growth, and gene transcription. Integrin signals not only connect the ECM to the cytoskeleton, but through a variety of adapter proteins and kinase actions, also initiate various intracellular signaling cascades, including the Ras/MAPK pathway, which results in the phosphorylation of the p42/44 MAPK, known as ERK (reviewed in Yee et al., 2008).



**Figure 4.15: LN both potentiates phosphorylation of early signaling intermediates essential to neurite outgrowth (ERK), and downregulates inhibitory signal transduction by decreasing the surface expression of p75NTR.** This decreases a key element of the receptor complex (NogoR/LINGO/p75NTR), which transduces myelin-derived inhibitory signals, and concomitantly depresses Rho activity. Furthermore the enhanced pool of PIP<sub>2</sub> resulting from increased PTEN activity binds profilin preventing it from sequestering G-actin monomers, that can instead be used in the construction of actin filaments for neurite outgrowth. This collection of LN induced effects converges to promote neurite regeneration in response to a permissive environment.

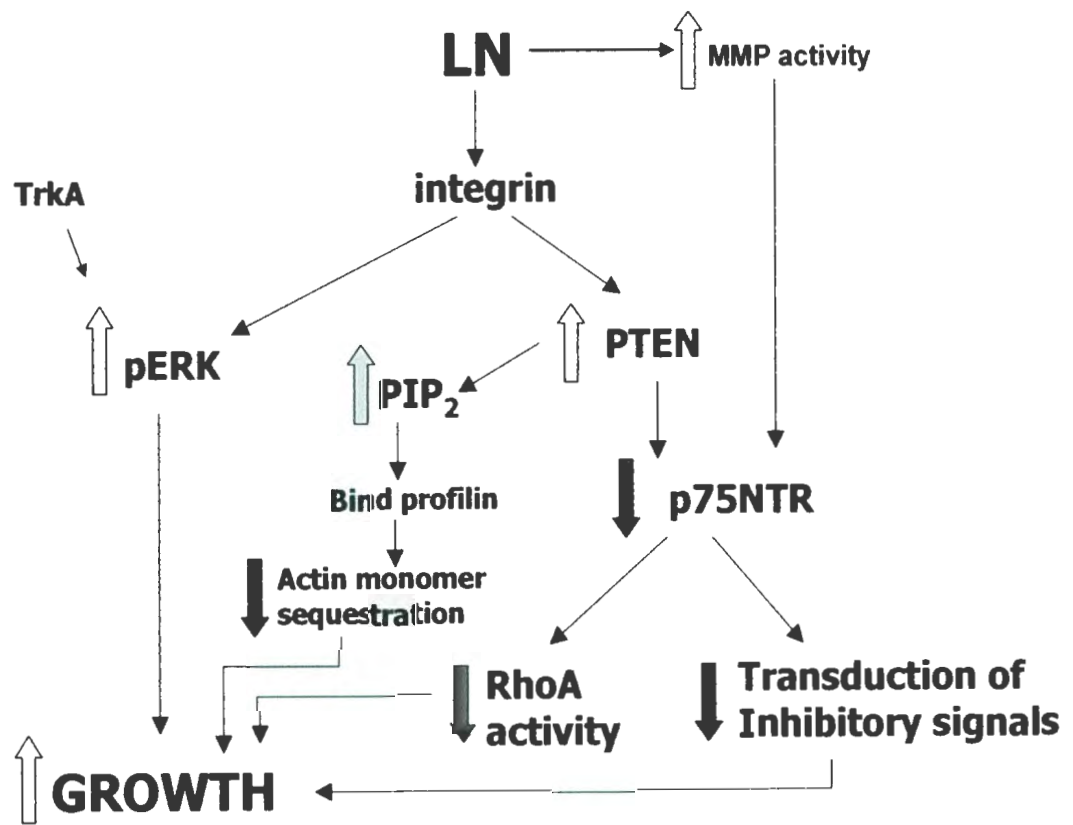


Figure 4.15

The phosphorylation of ERK is an early and essential signaling event for the initiation and extension of neurites in PC12 cells (Fukuda et al., 1995; Pang et al., 1995; Xiao and Liu, 2003). Inhibition of the phosphorylation of ERK in sympathetic neurons causes growth cone collapse (Atwal et al., 2003), and thus reduces neurite density (Creedon et al., 1996; Virdee and Tolkovsky, 1996). In this study, we demonstrate signaling crosstalk resulting in enhanced phosphorylation of ERK in response to co-stimulation with NGF and LN, greater than either stimulus alone. However, we also show that LN stimulation alone is sufficient for eliciting neurite outgrowth, suggesting that factors other than ERK activation contribute to the growth response.

NGF has been extensively studied in its capacity to modulate substrate adhesion and increase the capacity for PC12 neuritogenesis (reviewed in Fujii et al., 1982), by increasing the protein expression of integrin subunits (Danker et al., 2001; Rossino et al., 1990), and stimulating the accumulation of integrin  $\beta 1$  at the tips of filopodia in growth cones of sympathetic neurons (Grabham and Goldberg, 1997). While we failed to detect any upregulation effect of NGF on integrin  $\beta 1$  protein expression at 24 hours, we cannot rule out relocation of the existing integrin  $\beta 1$  protein.

More interestingly, few studies have investigated the effect of the ECM molecules on the expression of growth factor receptors. While the LN substrate did not influence the expression of TrkA, it was associated with a significant decrease in p75NTR (Fig. 4.3), which appears to be due to a combination of metalloprotease-mediated cleavage and a transcriptional downregulation (Fig. 4.4). p75NTR undergoes ectodomain shedding by a membrane metalloprotease, which releases a C-terminal fragment (CTF) that is rapidly further cleaved to yield a 25 kDa intracellular domain (ICD) fragment (Kanning et al.,



2003). Metalloprotease expression can be altered by ECM molecules, including LN (reviewed in Yong, 2005), but triple and not single neurotrophin exposure is necessary to potentiate regulated intramembrane proteolysis of p75NTR (Logan et al., 2006).

The role of p75NTR in the growth response is less clear, and may be cell type and context specific, although p75NTR is often associated with growth inhibition. Not only does BDNF act via p75NTR to inhibit axonal outgrowth in sympathetic neurons (Kohn et al., 1999), and antagonize NGF-mediated axonal outgrowth in both adult dorsal root ganglion (DRG) neurons (Kimpinski et al., 1999) and sympathetic neurons (Kohn et al., 1999), but a function blocking p75NTR antibody, REX, also directly enhances growth of sympathetic neurons elicited by NGF (Kohn et al., 1999). Finally, neurons deficient in p75NTR display more robust growth in response to Trk activation than wild-type neurons, and this growth cannot be inhibited by BDNF (Kohn et al., 1999). An examination of p75NTR knockout mice (Dhanoa et al., 2006; Hannila and Kawaja, 1999) revealed enhanced sympathetic innervation of the cerebellum in response to elevated levels of NGF, and enhanced sympathetic axonal arborization compared to wild-type. Mice deficient in p75NTR also display a higher spine density and greater complexity in hippocampal neurons than wild-type controls (Zagrebel'sky et al., 2005). ECM down-regulation of p75NTR expression levels could be mimicking the enhanced growth effect caused by genetic silencing.

Several studies have identified a role for p75NTR as the transducing element of a receptor complex that includes Nogo-R and LINGO1. This complex binds three known myelin associated inhibitors (MAI) of axonal regeneration: myelin associated glycoprotein (MAG), Nogo and oligodendrocyte myelin glycoprotein (reviewed in

Domeniconi et al., 2005). Since p75NTR siRNA has been shown to inhibit MAG-induced neurite retraction (Higuchi et al., 2003a), a reduction in p75NTR signaling is hypothesized to reduce the transduction of MAI signals that prevent axonal regeneration. In support of this hypothesis, neurons deficient in p75NTR are no longer responsive to myelin or individual MAIs (Wang et al., 2002a), and cleavage of p75NTR in DRG neurons disinhibits outgrowth and promotes branching of neurites in the presence of myelin (Ahmed et al., 2006). Furthermore, LN is able to prevent MAG inhibition of NGF-induced Rac activation, and thus override the inhibitory cues (Laforest et al., 2005). The removal of p75NTR from the cell surface provides a potential mechanism for this phenomenon.

The inhibitory effects of MAI on axonal growth, transduced by p75NTR, are mediated by the intracellular activation of Rho GTPase (Yamashita et al., 2002; Yamashita et al., 1999), and subsequent activation of the Rho-ROCK signaling cascade (Alabed et al., 2006). In its active GTP-bound state, Rho inhibits axonal elongation and mediates growth cone collapse (Gehler et al., 2004; Nikolic, 2002) by interacting with proteins that regulate the assembly and stability of the actin cytoskeleton (Kaplan and Miller, 2003). Inactivation of Rho or ROCK is sufficient to allow neurite formation on myelin substrates (Kaplan and Miller, 2003; Yamashita et al., 1999), and facilitates regeneration after axonal injury (Ahmed et al., 2005; Gehler et al., 2004). Rho is constitutively activated by unliganded p75NTR (Yamashita et al., 1999), a relationship that is negated by neurotrophin binding. Unoccupied p75NTR negatively regulates filopodial dynamics (Gehler et al., 2004), and thus negatively impacts neurite outgrowth. Our study demonstrates that LN decreases p75NTR expression, which mediates a



decrease in the activation of Rho (Fig 4.7), and could thus promote growth. A similar effect has been demonstrated using siRNA against p75NTR, which decreases active Rho and results in a subsequent growth response (Ahmed et al., 2005). Furthermore, we also show that the overexpression of p75NTR results in inhibition of neurite growth, and sustained activation of Rho; the inhibitory effects can be blocked by the presence of the Rho kinase inhibitor (Fig. 4.7B,C).

The LN-mediated downregulation of p75NTR is associated with an upregulation of PTEN (Fig. 4.9). In addition to its well-known role as a tumor suppressor, recent studies reveal a role for PTEN in the regulation of multiple cellular functions, including cell size, migratory behaviour, modulation of the actin cytoskeleton (von Stein et al., 2005) and growth (reviewed in Sulis and Parsons, 2003). In this regard, PTEN also has a role in neural development, particularly neurite extension, and the suppression of PTEN expression impairs neuritogenesis. (Fig 4.11, (Lachyankar et al., 2000).

As a dual specificity phosphatase, PTEN is capable of dephosphorylating both proteins and lipids. The lipid phosphatase activity of PTEN functions primarily in the negative regulation of PI3K activity. Selective activation of PI3K results in defective neurite outgrowth in PC12 cells (Ashcroft et al., 1999). Furthermore, recent studies utilizing a lipid phosphatase null mutant of PTEN demonstrated that loss of this domain, and its subsequent functions, impaired neurite outgrowth from primary cortical neurons; an effect hypothesized to be associated with the effect of PTEN on tau phosphorylation and subsequent alteration of microtubule interactions (Kerr et al., 2006; Zhang et al., 2006a).

The upregulation of PTEN in response to LN may result in multiple other signaling events in the cell that have direct effects on neurite outgrowth. PTEN hydrolyzes phosphoinositide  $PI(3,4,5)P_3$  to produce second messenger  $PI(4,5)P_2$ , thus increasing the available pool of  $PI(4,5)P_2$  and decreasing the downstream activation of  $PI(3,4,5)P_3$  targets, including Akt and GSK (Beckner et al., 2005). Additionally,  $PI(4,5)P_2$ , which is increased in response to PI3K inhibition, can bind profilin, a protein that functions to sequester G-actin monomers. The binding of profilin by  $PI(4,5)P_2$  releases G-actin, increasing the available monomers for actin filament building and associated neurite extension (reviewed in Witke, 2004).

In summary, we propose a signaling model (Fig 10) wherein LN upregulates PTEN, which can impact neurite outgrowth through numerous effectors, but perhaps importantly, through the downregulation of p75NTR, a receptor that is implicated in neurite growth inhibition in a variety of neuronal cell types. Thus, by decreasing surface expression of p75NTR, LN results in the decreased transduction of multiple inhibitory growth pathways, both myelin-derived and Rho associated. (Figure 4.15). LN is upregulated following peripheral nerve injury (Wallquist et al., 2002), and likely plays a physiological role in regeneration, potentially by engaging in a combination of enhanced growth programs and over-riding inhibitory cues.



## **Chapter 5: A method to assess multiple aspects of the motile behaviour of adherent PC12 cells on applied biological substrates**

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### **5.0 Summary**

Cellular migration is central to a wide range of biological and pathological processes *in vivo*. *In vitro* cell migration assays can be used to obtain invaluable information relating to the mechanism of cell movement, but current available methods can be limiting. Here we describe a novel motility assay that allows the simultaneous investigation of both quantitative and qualitative aspects of a population of motile cells as they move across a variety of substrates. By plating cells in a confluent monolayer on a coverslip, the monolayer can then be inverted to migrate over a larger substrate-coated coverslip, which can subsequently be reliably quantified, and subjected to immunocytochemistry and confocal imaging. This assay can be used to assess multiple aspects of motility, including distance, quantity, morphology, polarization and component colocalization. To demonstrate the utility of this assay, it was applied to the study of a stimulator of PC12 cell migration, nerve growth factor (NGF), and how this migration is influenced by the extracellular substrate, laminin. Furthermore, since mutations to the NGF receptor, TrkA, have been noted to alter the behaviour of PC12 cells in response to NGF, a PC12 subline that expresses a mutated TrkA receptor was utilized to illustrate that a Y785F mutation in the cytoplasmic tail of TrkA results in increased migration in response to the stimulus compared to the control PC12s.

### **5.1. Introduction**

Cell migration plays an essential role in embryonic development and is of crucial importance to multiple physiological processes, including wound healing, angiogenesis, the immune response and inflammation (for review, see (Lauffenburger and Horwitz, 1996). Furthermore, cellular motility is central to certain pathological conditions such as tumour invasion and metastasis (for review, see (Engers and Gabbert, 2000; Ervin and Cox, 2005).

Cell movement follows a characteristic pattern of events, beginning with the protrusion of a leading edge lamellipodium, followed by translocation of the cell body and finally retraction of the trailing edge (for review, see (Howe, 2004). These steps are physically and spatially distinct, and are regulated by a complex myriad of intracellular biochemical events which ultimately influence the multiple components of the cytoskeleton. These processes are dynamically regulated to allow the cell to efficiently alter the direction or speed of movement, as each motile cell is continually changing position with respect to other cells and the surrounding extracellular environment (for review, see Vasiliev, 2004).

Key modulators of cell motility include the extracellular matrix (ECM), and cytokines/growth factors (Strachan and Condic, 2004). A family of receptors known as integrins mediates signals from the ECM. When activated by ECM ligands, integrins aggregate to form focal adhesions which directly influence the actin cytoskeleton, often resulting in cell movement (Kanda et al., 2004). Growth factor signals are also mediated by various surface receptors; for example, nerve growth factor (NGF) signals are mediated by both the high affinity receptor TrkA, and the low affinity p75NTR. The



TrkA receptor is activated upon ligand binding, and activation is associated with the phosphorylation of individual tyrosine residues on the cytoplasmic tail of the receptor (for review, see (Kaplan and Miller, 2000)). Phosphorylation of the tyrosine residue at position 490 (Y490) activates a Ras-ERK cascade, while the tyrosine residue at position 785 (Y785) interacts with phospholipase C (PLC- $\gamma$ ) to activate the PLC- $\gamma$ -PKC-ERK signalling pathway. Both of these pathways result in the activation of ERK (Loeb et al., 1994; Obermeier et al., 1993a; Stephens et al., 1994), which is known to play a vital role in the motility of PC12 cells (Ho et al., 2001; Ho et al., 2005). A previous study revealed that cells expressing mutations of TrkA at Y490 and Y785 responded differently than wild-type PC12 cells to various combinations of NGF and laminin (Rankin et al., 2005). In order to pursue our investigation of the ECM and NGF in regulating cell behaviours, it was necessary to establish a migration assay that could provide both quantitative and qualitative information regarding the motile behaviour of these cells.

Investigation of cellular motility fundamentals *in vitro* is thought to provide insights into diverse *in vivo* biological processes, including tissue formation and tumour metastasis, and is essential to the development of pharmacological approaches that attempt to control these phenomena. Currently available motility assays do not afford the option of using surface applied biological substrates, studying motility over long time periods, or allowing both quantitation and qualitative examination of cellular movement at once, thus limiting their applicability to the study of substrate-receptor interactions in motility. This study presents a novel motility assay that quickly and easily provides information regarding distance, morphology, quantity, polarization and component colocalization in an easily quantifiable system.



To illustrate the utility of this assay, we examined the importance of the TrkA mutation to cellular motility on two different substrates. For illustrative purposes, we have focused solely on a highly motile mutation, the Y785F cell line. The approach outlined here would also be useful to the following types of studies:

- (I) Quantitative assay of adherent cell motility.
- (II) Qualitative assay of adherent cell motility, including morphological changes and component polarization.
- (III) Assessment of various inhibitors/mutations on cellular motility.

## **5.2 Materials and Methods**

### **5.2.1 Cell Culture Model**

The experimental model consisted of wild-type rat pheochromocytoma (PC12) cells, and a mutated PC12 derivative cell line, Y785F (gifts from Dr. David Kaplan, Hospital for Sick Children, Toronto, ON). The Y785F cell line was created by Stephens et al. (1994). Briefly, a PC12 cell line that lacks a functional TrkA receptor (PC12nnr5) was transfected to express a TrkA receptor that had been altered via site-directed mutagenesis to abrogate the Y785 autophosphorylation site of the cytoplasmic tail.

Cell lines were maintained on rat-tail collagen coated tissue-culture flasks in RPMI 1640 medium (Invitrogen, Burlington ON) supplemented with 10% horse serum (Invitrogen), 5% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin/glutamine solution (Invitrogen). Cells were incubated at 37°C in 5% CO<sub>2</sub>, and cultured to 80% confluence prior to trypsinization for subculturing purposes.

## **5.2.2 Detailed Procedure**

See Figure 5.1 for schematic diagram of procedure.

### **5.2.2.1 Coverslip Preparation**

In order to prepare the coverslips so that PC12 cells would adhere to the glass, they were first pre-coated on one side with poly-D-lysine (PL, BD Biosciences, Mississauga, ON). To accomplish this, 12 mm diameter glass coverslips (3 per experimental condition; Carolina Biologicals, Ottawa ON) were carefully spread over a piece of sterile filter paper that had been cut to fit inside of a 150 mm tissue culture dish. All coverslip manipulations were performed using sterile fine point tweezers within a biosafety cabinet to avoid introducing contamination into the cultures.

Once coverslips were adequately separated, PL (40  $\mu\text{g/ml}$ ) was applied dropwise to the upward face of the coverslip, relying on surface tension to keep the liquid as close to the edges as possible. The tissue culture dish lid was gently placed on top to reduce evaporation. After 30 min, PL was aspirated and the coverslips were allowed to dry before a second coat was applied. After 30 min, the PL was again aspirated and coverslips were allowed to dry prior to rinsing with sterile distilled water, and air-drying once more. This coating method ensured that cells remained adherent to only one side of the coverslip.

The PL-coated 12 mm coverslips were then placed, one per well, into a 24-well plate with the PL-coated side facing up.

**Figure 5.1: Procedure Schematic.** A. 12-mm coverslip (clear circle) is coated on one side with PL. B. PC12 cells (black circles) are plated onto the PL-coated coverslip and allowed to settle and synchronize overnight. C. 12-mm coverslip is inverted and placed cell-side down onto a PL- or LN-coated 18-mm coverslip (cross-hatched), which serves as the migratory field. D. Over a period of 24-48 h, cells are allowed to migrate out from underneath the overlaid coverslip. E. After photographing, the 12-mm coverslip is removed and the remaining migratory cells are subjected to immunocytochemistry.



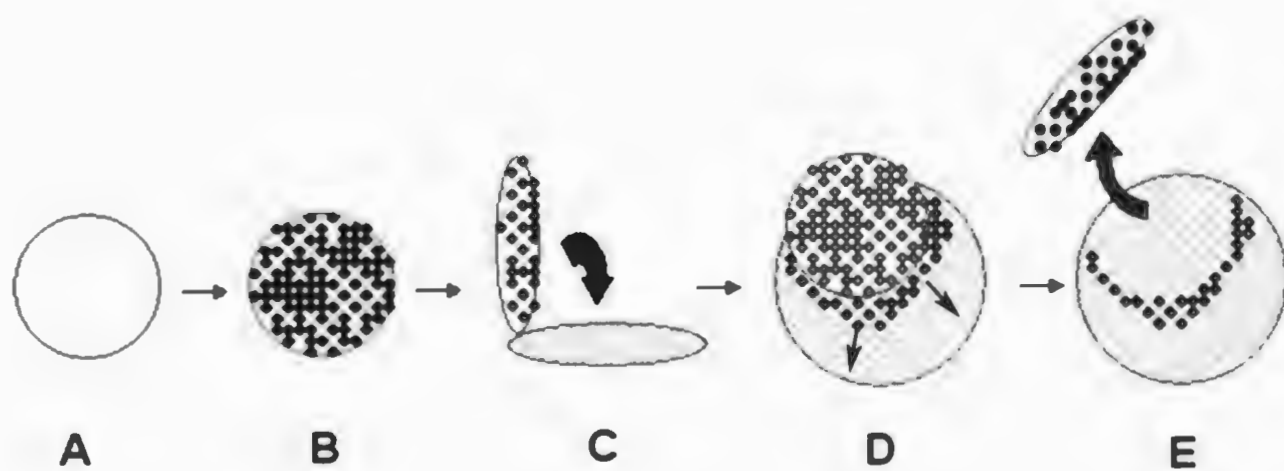


Figure 5.1

#### **5.2.2.2 Preparation of the Cells**

Cells were trypsinized using trypsin-EDTA (Invitrogen) in order to release them gently from the surface of the tissue culture flask. The trypsin reaction was stopped by the addition of serum-enriched RPMI 1640 medium, and cells were subsequently pelleted from the suspension by centrifugation (1000 rpm, 5 min). At this stage, the supernatant was aspirated to leave the cell pellet, which was then resuspended in serum-free RPMI. Cells were loaded onto the coverslips by pipetting between  $1.5 \times 10^5$  and  $2.5 \times 10^5$  cells into each well of the 24-well plate, ideally diluted to a final volume of 0.5 ml. This ensured 90-95% confluence. Cells were allowed to settle overnight (16 h) at 37°C, 5% CO<sub>2</sub>. The serum free environment was designed to synchronize the population and reduce proliferation rates.

#### **5.2.2.3 Preparation of the Migration Substrate**

The following day, 18-mm diameter round glass coverslips (1 per 12-mm coverslip; Fisher Scientific, Fairlawn NJ) were undercoated with PL (1 coat x 30 min; see step 2.2.1), and allowed to dry. 18-mm coverslips were then separated into two groups: half of the coverslips were treated to a second coat of PL (30 min) and allowed to dry as before prior to washing with sterile distilled water. The other half of the coverslips was treated to 2 coats of laminin (LN, 25 µg/ml, Invitrogen) for 30 min each, allowing the coverslips to dry in between coats.

The coated 18-mm coverslips were placed, 1 per well, within a 12-well plate, coating side facing upwards.

#### **5.2.2.4 12-mm Coverslip Inversion**

1 ml of serum-enriched RPMI 1640 medium was added to each well of the 12-well plate, and sterile tweezers were used to push out any air bubbles that may have become trapped underneath the 18-mm coverslip. This plate was set aside, and the 24-well plate (containing cells on 12-mm coverslips) was removed from the incubator for preparation. Following aspiration of the media from the 24-well plate wells, the cell-covered coverslips were washed twice with fresh medium in order to remove excess and non-adherent cells, ultimately replacing 0.5 ml of fresh medium to the 24-well plate. The 12-mm coverslips were next gently removed from the 24-well plate by using a sterile needle bent to a 90° angle to raise the edge of the coverslip so that it could be gripped with the sterile tweezers. The 12-mm coverslip was then inverted and gently lowered, cell side down, onto the top of the 18-mm coverslip in the 12-well plate, moving slowly to ensure no air bubbles become entrapped between the glass coverslips that may damage the monolayer or interfere with microscopic visualization.

Once the 12-mm coverslip was in position on top of the 18-mm coverslip, the medium was changed in the well to remove any floating debris that may have settled in the migratory field. This medium change also provided the option of adding a stimulus or inhibitor of motility to the culture medium. For the experimental conditions investigating the effects of NGF, NGF (Harlan, Indianapolis IN) was added to the culture medium at a concentration of 50 ng/ml. Once fresh medium was replaced in the well, the coverslips were examined using a light microscope to ensure the edges of the coverslip were clear of cellular debris. This condition was photographed as a “time zero” control, and plates



were incubated at 37°C, 5% CO<sub>2</sub> for the migratory time period of choice, generally 24-48 h.

#### **5.2.2.5 Collection and Analysis of Quantitative Data**

At the scheduled time point (24 or 48 h), plates were removed from the incubator and an inverted microscope fitted with a digital camera was used to photograph individual fields of view of the overlapping edges of the two coverslips in the well. These images were used to assess the various parameters of cellular motility: individual cells were either manually counted within each imaged field of view, or alternatively quantitated automatically using particle recognition software, such as Kodak™ Image Analysis Software (Molecular Imaging Systems, New Haven CT). Values were averaged per experimental condition to provide a relative quantity of cell motility for comparison. Statistical analysis was performed using Graph Pad Prism 4 (Graph Pad Software Inc., San Diego CA), with significance being determined using one-way ANOVA testing.

The distance of migration can be assessed using an ocular micrometer to measure distance travelled. Alternatively, distance measurements can be derived retrospectively from the images. These images could also be used to assess the morphological stages as the cells migrated.

#### **5.2.2.6 Immunocytochemistry**

In order to subject the migratory cells on the 18-mm coverslip to immunocytochemistry, it was first necessary to carefully remove the 12-mm coverslip from the surface. This was accomplished using an aspirator fitted with a 3-mm hose as a

vacuum device. The aspirator was also used at this point to remove all remaining media from the well.

18-mm coverslips were washed with phosphate buffered saline (PBS; Invitrogen) for 5 min. PBS was subsequently aspirated from the wells and replaced with 4% formaldehyde (Fisher Scientific) for 20 min for fixation purposes, and once again washed with PBS for 5 min. Cells were next permeabilized by incubation with 0.1% Triton-X (Sigma, St. Louis MO) for 10 min, washed in PBS for 5 min, and blocked in 10% goat serum in PBS for 1 h. At this point, coverslips were placed cell-side-up on glass slides within a humidifying chamber. Primary antibodies directed against integrin  $\beta 1$  (1:250, polyclonal, Chemicon, Temecula CA), and total tubulin (1:1000, Sigma, monoclonal) were applied dropwise to the coverslips, just covering the cells. The humidifying chamber was subsequently placed at 4°C, and cells were allowed to incubate overnight (16 h).

The following morning, 18-mm coverslips were placed in 35-mm tissue culture dishes of PBS and allowed to wash for 10 min prior to incubation of the cells in Cy2 and Cy5 tagged secondary antibodies (1:100, Jackson ImmunoResearch Laboratories Inc., West Grove PA) for 1 h. Following a final 10 min wash in PBS, 60  $\mu$ l of glycerol was placed onto the surface of a clean glass microscope slide (Fisher Scientific) and the 18-mm coverslip was inverted and placed cell-side-down onto the glycerol. Excess glycerol was wicked away using filter paper, and edges of the coverslip were sealed using clear nailpolish.

Slides were visualized using confocal scanning laser microscopy, and staining patterns were assessed based on the direction of locomotion.

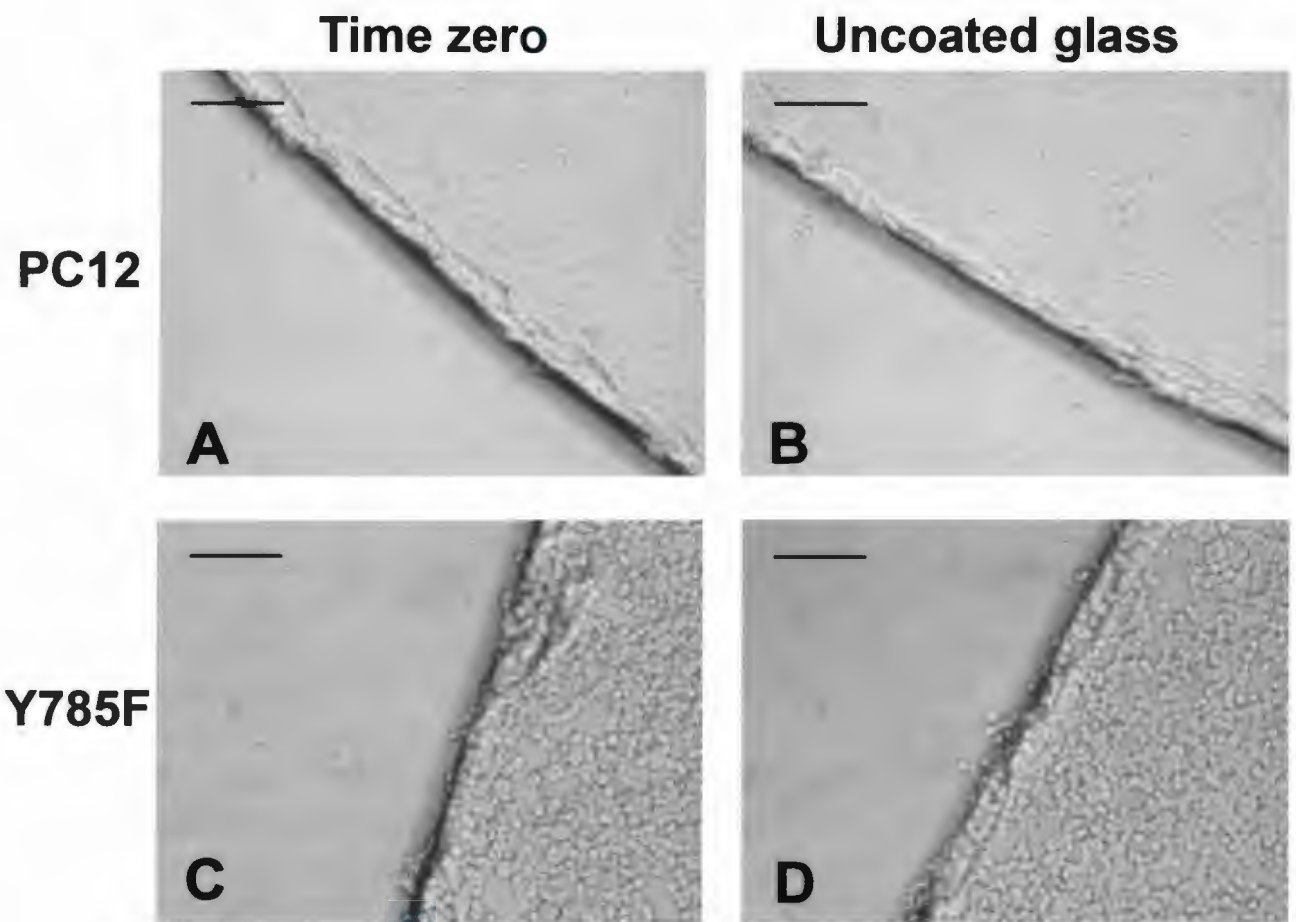
### 5.3 Results

PC12 cells and derivative mutants (Y785F) plated onto PL-coated coverslips and inverted onto an adhesive substrate (PL or LN), were induced to migrate across the substrate, thus moving out from underneath the overlaid coverslip, exposing a population of motile cells to study. This protocol was optimized using uncoated 18-mm glass coverslips as the migratory surface in lieu of an adhesive substrate (Fig. 5.2). This served as a negative control because the PC12 cells do not attach well to glass (Greene et al., 1998); they are thus unlikely to locomote away from the PL coating of the overlaid coverslip, as noted by the lack of migration visible after 24 h in Figures 5.2B and 5.2D. The addition of NGF to the culture medium has been shown to increase the rate of cell movement in PC12 cells (Ho et al., 2001; Ho et al., 2005), and thus this experimental condition also served as a positive control for cellular motility (Figs. 5.3C and F for PC12, Fig 5.4C and F for Y785F).

In order to compare the effects of different biological substrates on cell motility, the cell-covered coverslip was inverted onto one of two substrates (PL or LN), and a digital photo was taken at the starting point, time zero. Fig 5.3A and D are representative images taken at time zero, illustrating the clean, confluent edges of the overlaid coverslip and the initial lack of PC12 cells in the migratory field. Fig 5.3B and E are representative of field of view (FOV) images used for the comparison of relative motility on the two different substrates, PL (Fig 5.3B) and LN (Fig 5.3E). As illustrated by the relative quantity of cells emerging from the confluent edges of the coverslips, the type of substrate available in the migratory field appears to influence the relative quantity of



**Figure 5.2: Negative Controls.** Photomicrographs illustrate negative controls for optimization of procedure. Panels A and B show PC12 cells, and panels C and D show Y785F cells that have been plated on PL-coated 12-mm coverslips, and inverted onto uncoated 18-mm glass coverslips. Images are taken at time zero (A,C) and 24 h (B,D) to illustrate that there is no movement in the absence of an adhesive substrate. Scale bar = 200  $\mu\text{m}$ .



**Figure 5.2**

**Figure 5.3: Effect of Substrate on PC12 Cells in the Presence or Absence of NGF.**

Photomicrographs illustrate PC12 cells plated on PL-coated 12-mm coverslips, and subsequently inverted onto substrates of PL (A-C) or LN (D-F), in the absence of NGF (B,E) or in the presence of NGF (C,F). Note the relative increase of motile cells on LN relative to PL, and the various cellular morphologies. Scale bar = 100  $\mu\text{m}$ .



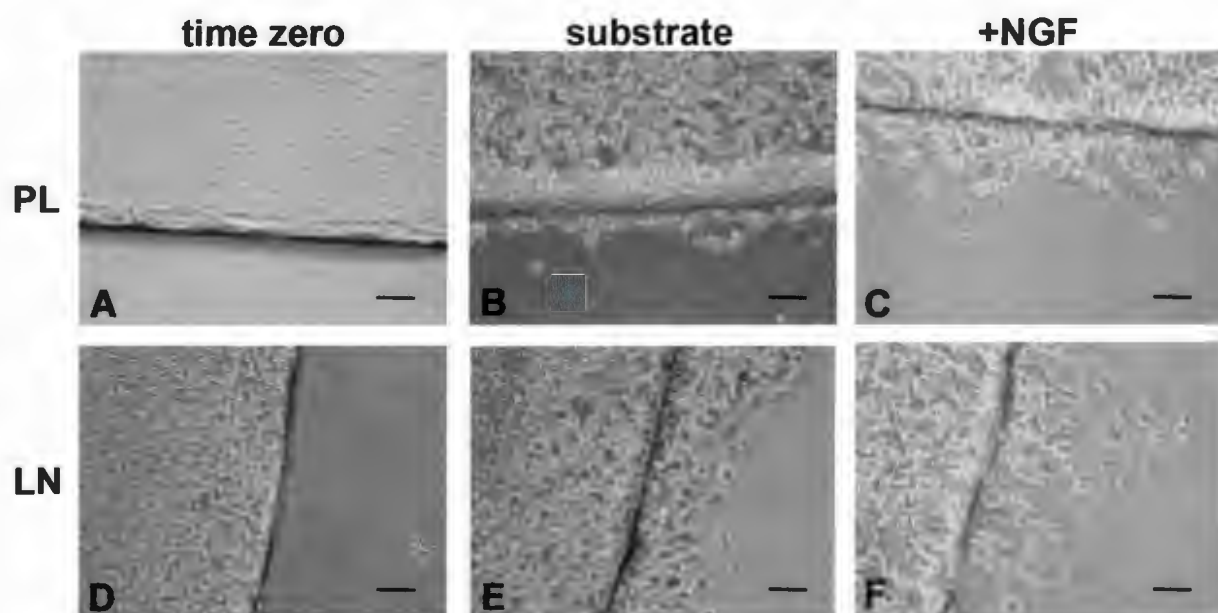
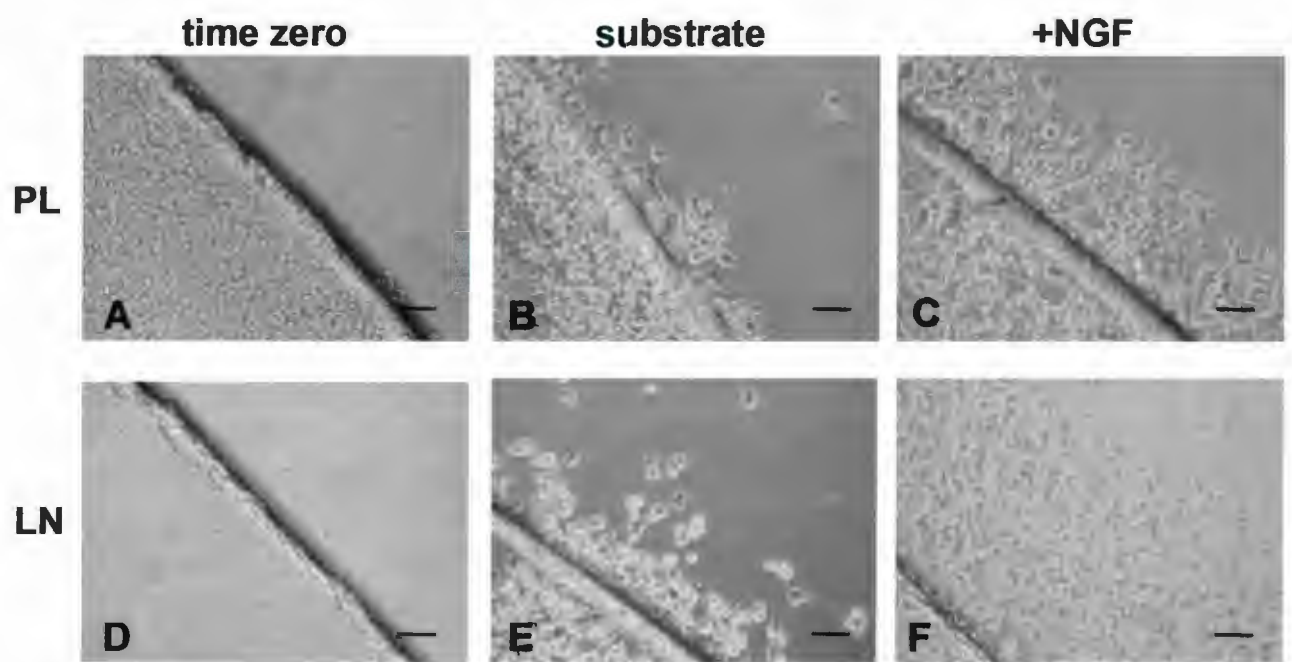


Figure 5.3

**Figure 5.4: Effect of Substrate on Y785F Cells in the Presence or Absence of NGF.**

Photomicrographs illustrate Y785F cells plated on PL-coated 12-mm coverslips and subsequently inverted onto substrates of PL (A-C) or LN (D-F), in the absence of NGF (B,E) or in the presence of NGF (C,F). Similar to Figure 2, note the relative increase of motility on LN relative to PL, and the various cellular morphologies. Scale bar = 100  $\mu\text{m}$ .



**Figure 5.4**

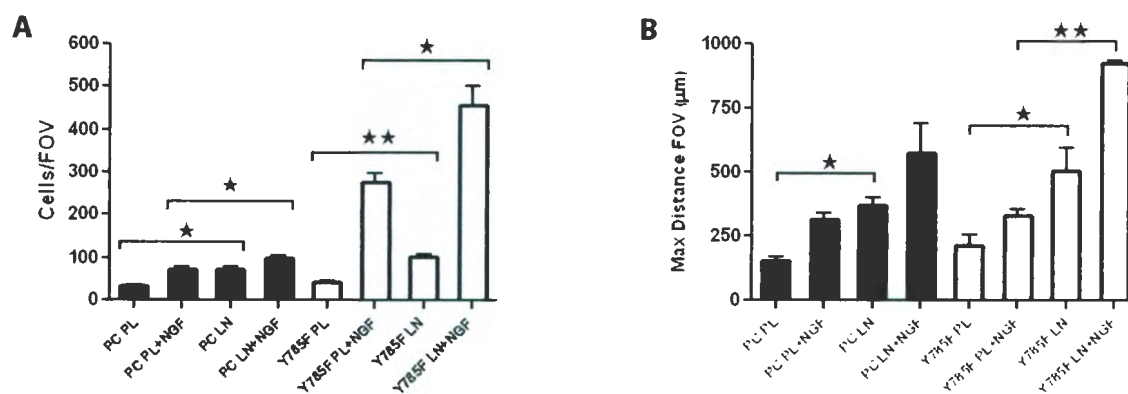


migration across the migratory field. It is also possible to note cellular morphology of the motile cells in these images (Figs 5.3 and 5.4). The addition of NGF (50 ng/ml) to the culture media also influenced the motile behaviour of the PC12 cells. Fig 5.3C and F are representative images for the comparison of relative motility on the different substrates, PL (Fig 5.3C) and LN (Fig 5.3F) in the presence of NGF. Again it appears that the addition of NGF increased chemokinesis. The experimental condition that exhibited the most motility in wild-type PC12 cells involved cells migrating across the laminin substrate in the presence of NGF. An examination of the morphology reveals that the cells are spreading on LN, extending processes and leading edge protrusions.

To examine the effects of the Y785F mutation of the TrkA receptor on motile behaviour, similar experiments were performed using the mutated cell line. The representative time zero images showed clean, confluent edges of the overlaid coverslip on both substrates (Fig 5.4A and C). The cells showed increased migration on LN compared to PL (Fig 5.4B and E), while NGF increased the chemokinesis of these cells on both PL and LN (Fig 5.4C and F).

Quantitative analysis of the migratory behaviour consisted of counting the number of cells that moved out from underneath the overlaid coverslip and into the migratory field of view. This analysis revealed that PC12 cell motility is significantly increased on a LN substrate relative to the synthetic PL substrate (Fig 5.5A). The addition of NGF also resulted in a significant increase in motile behaviour. The PC12 cells expressing the Y785F TrkA mutation are also significantly more motile than wild-type PC12 cells, particularly when exposed to a LN substrate or NGF (Fig 5.5A). Fig 5.5B represents graphical comparison of the maximum migratory distance determined by ocular

**Figure 5.5: Quantitation of Relative Motility.** A. PC12 cells (black bars) treated with NGF (50 ng/ml) exhibit increased cellular motility. Cells exposed to a LN substrate also exhibit increased cell motility relative to the PL substrate. The combination of LN and NGF resulted in significantly more motility than either condition alone. In relation to wild-type PC12 cells, Y785F cells (white bars) demonstrated an increased rate of motility in response to NGF or LN, which increased significantly upon exposure to both stimuli (\* $p < 0.01$ , \*\* $p < 0.001$ ). B. Graphical comparison of maximum migratory distance determined by ocular micrometer measurement. Distance traveled is enhanced by the addition of NGF (50 ng/ml) or exposure to a LN substrate. The combination of NGF and LN resulted in a trend of increased distance traveled than either condition alone (\* $p < 0.01$ , \*\* $p < 0.001$ ).



**Figure 5.5**

micrometer measurement. The addition of NGF to culture medium enhanced the maximum distance travelled, as did exposure to the LN substrate. Interestingly, the combination of NGF and LN resulted in enhanced distance travelled than either condition alone.

By subjecting the 18-mm coverslips to immunocytochemistry, it was possible to examine cellular components of the motile cells moving across the migratory field. For this experiment, cells were co-stained with antibodies directed against total tubulin and integrin  $\beta 1$ , and detected with fluorescently tagged secondary antibodies. The total tubulin and integrin  $\beta 1$  stains were visualized using confocal laser scanning microscopy to examine the cellular morphology of the motile cells (Fig 5.6). The cells appear to be developing lamellipodia and leading edge protrusions. A closer examination of the confocal images revealed a polarization of integrin  $\beta 1$  to the leading edge of the cellular processes, relative to the total tubulin localization (Fig 5.6). This pattern of component localization was consistent between both the wild type PC12 cells and the derivative PC12 cells expressing the Y785F mutation of the TrkA receptor.

## **5.4 Discussion**

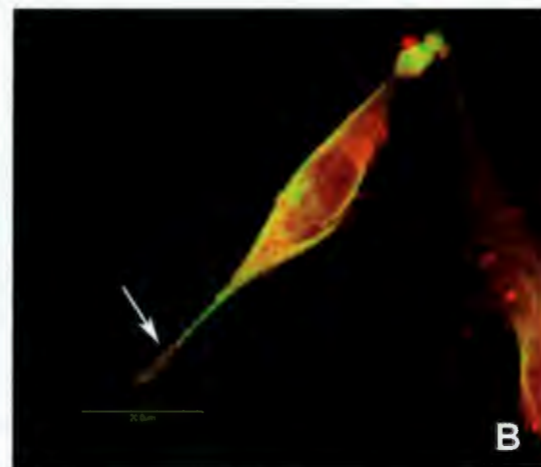
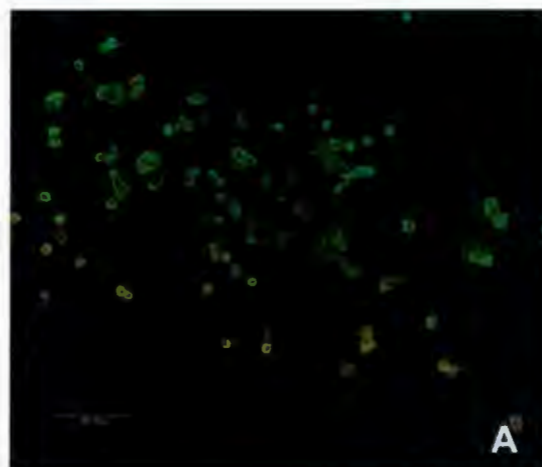
### **5.4.1 Overall Assessment of the Protocol**

This study presents a relatively simple motility assay that allows the simultaneous study of multiple aspects of cellular movement, including distance, direction, quantity, morphology of the intermediate stages of movement and component polarization, with



**Figure 5.6: Integrin  $\beta 1$  Polarization.** Confocal images representing PC12 cells (A, B) and Y785F (C, D) plated on PL-coated 12-mm coverslips and allowed to migrate onto a LN substrate. Cells were stained with integrin  $\beta 1$  (red) and total tubulin (green) and visualized with Cy2 and Cy5 tagged secondary antibodies. Note the dense aggregation of integrin  $\beta 1$  in the protruding leading edges (white arrows). A, C: scale bar = 100  $\mu\text{m}$ . B, D: scale bar = 20  $\mu\text{m}$ .

PC12



Y785F

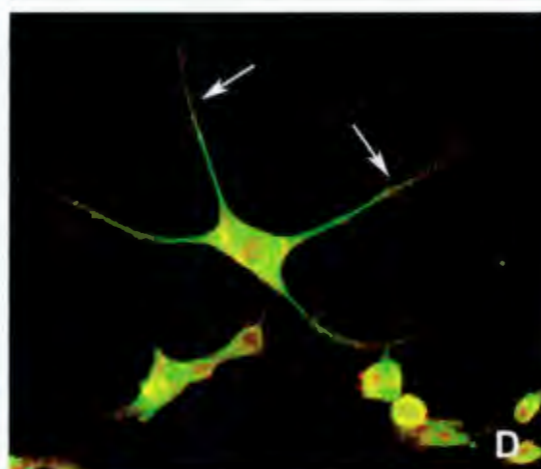


Figure 5.6

relatively low expenditure of time, space and materials. This assay allows the investigation of multiple parameters, such as cellular mutation and pharmacological inhibition of intracellular signalling intermediates, and allows the assessment of their effects on motility. Furthermore, the assay can be permuted to allow the use of any combination of coatings and substrates. Though numerous motility assays exist, to our knowledge, this is the only method that allows accurate quantitation and qualitative assessment of a population of motile cells across an artificially applied substrate (for example, LN coating) without inflicting scratches or damaging the migratory substrate or the cells in any way. In addition, the option of automated quantitation using particle recognition software reduces the time typically associated with analysis of motility assays, most of which require counting manually for accuracy. In this study, we have identified clear differences in the motility of wild-type PC12 cells and a derivative expressing the Y785F TrkA mutation. Because the phosphorylation of the Y785 residue is associated with an intracellular signalling pathway involving PLC- $\gamma$  and PKC, this information can provide clues as to the regulation of chemokinetic behaviour. Further investigation of these pathways is the subject of ongoing investigation.

#### **5.4.2 Troubleshooting**

With meticulous care and gentle coverslip handling, this protocol can be carried out with minimal damage to the cellular monolayer. However, due to the necessary manipulations/inversions of the coverslips, this protocol is suited only to the study of strongly adherent cells. Cells may become dissociated from the coverslip if the coverslip is initially overloaded. Should this be the case, the option exists to plate the cells less

confluently, and allow them to grow confluent over the coverslip prior to inversion.

Furthermore, it is essential to gently rinse the monolayer and change the media in the final well to remove any cells that do become dislodged and may settle in the migratory field thus confounding counts.

The initial step of plating the cells on a PL-coated coverslip and flipping it onto a LN-coated migratory field is key to this assay. It serves to confine the cells to a specific circular area without damaging the LN substrate, thus forcing the cells to migrate radially out from under the coverslip. The edge of the coverslip then serves as a definitive starting line, lending accuracy to the quantitation. Additionally, by confining the cells as such, it allows us to monitor population movement, rather than individual cell movement. Plating the cells directly onto the LN-coated coverslip would allow the study of individual cell movement, but the non-directional nature of migration in the absence of a chemotactic source would prevent the study of population movement, and eliminate the quantitation available with the dual coverslip system.

It is also imperative to avoid the entrapment of air bubbles beneath the coverslips that may damage the cellular monolayer and interfere with microscopic visualization. To this end, coverslips that are coated with cells should not be placed into a dry well, but instead be lowered gently into a medium-containing well.

If the weight of individual coverslips varies widely, it is possible that the weight of the coverslips could differentially influence the number of cells migrating. In our experiments, it is unlikely that the weight of the coverslip is influencing the number of cells emerging from beneath the glass, as the weight of the individual coverslips ranged no more than 3% of total weight. Notwithstanding, it is prudent to replicate experimental



results to eliminate the potential influence of differential coverslip weights. The present experiments were replicated three times without significant differences in results.

This motility assay is likely affected by similar factors that limit the reliability of the traditional wound healing assays: proliferation is possibly playing a role in the increased number of cells noted in the migratory field. We addressed this issue by synchronizing the cell population in a serum deprivation state prior to beginning the migration. This issue could also be addressed by low-level irradiation or the addition of anti-mitotic agents to the culture medium.

#### **5.4.3 Alternative and/or Support Protocols**

This assay can be permuted in a number of different ways allowing for the use of different coverslip coatings and substrates to investigate their effects on motility. Additionally, alternate cytokines/ growth factors or pharmacological inhibitors can be added to the culture media of migrating cells to assess their effects on motility. Furthermore, it is possible to derive even more information from this assay by altering the endpoint; for example, rather than proceeding to immunocytochemistry, the cells can be scraped from the coverslip and the protein harvested for Western blot analysis of cellular components. Furthermore, a population of cells could easily be marked and followed over a finite period of time to determine the rate or speed of motility.

As it stands, this assay measures chemokinesis (random movement), but can easily be converted to a chemotactic (directed migration) assay by the addition of a neurotrophin-soaked plug (for review, see Entschladen et al., 2005) to one end of the well to create a concentration gradient for cells to migrate towards.

In general, cellular motility can alternately be assessed using colloidal gold particles to track movement of individual cells, which clear a path by phagocytosing synthetic bead substrate (for review, see Linger, 2003; Staton et al., 2004; Windler-Hart et al., 2005). This method provides an accurate assessment of the distance and route a cell takes, but the importance of the substrate to this assay doesn't allow for permutations to test alternate biological substrates. Also, population movement is harder to assess, which is potentially important because cellular relation may influence motility.

Motility can also be assessed using a wound-healing assay in which a confluent cell monolayer is wounded by using a micropipette tip to denude an area of cells. This wound is then monitored microscopically to visualize the migration of cells into the scratch (for review, see Entschladen et al., 2005). This assay is rapid and simple, but reliable quantification using a traditional scratch assay can be difficult due to differences in the precision of the initial scratch and the lack of a definitive starting point for movement. The wound-healing assay tends to damage the cells lining the edges of the wound, and results in floating debris that must be removed. This assay has been updated recently to include several variations designed to avoid the aforementioned pitfalls of the traditional method. Current scratch methods employ electrical current (Keese et al., 2004), polyelectrolyte patterns (Kumar et al., 2005) or mechanical equipment (Yarrow et al., 2004) to standardize scratch size and avoid damage to edges of the monolayer. Unfortunately the scratch assay continues to eliminate the possibility of studying migration on alternate substrates, which can also be damaged or scratched off when the surface is scratched. Moreover, it is difficult to study motility over longer time points, as the scratch can entirely fill in and be disguised in the well.



Migration and invasiveness can also be quickly quantified using a transwell migration assay, including the Boyden Chamber, in which cells are plated on one side of a porous membrane and allowed or induced to migrate to the other side of the membrane over a 4-6 h time period (Ervin and Cox, 2005; Ho et al., 2001; Ho et al., 2005; Staton et al., 2004; Tezel et al., 2001; Windler-Hart et al., 2005). While this assay can accurately determine chemotactic versus chemokinetic stimulation, and allows screening of multiple substances for motile influence, it does not allow any analysis of cells in motion. This assay can be technically difficult and time-consuming, both in terms of set up and analysis, and accuracy of the counts can be problematic, particularly when only a small number of cells pass through the filter (for review, see Staton et al., 2004). Furthermore, automated analysis is often confounded by the inability of software to differentiate between a migrated cell and a pore in the membrane, although manual analysis can provide accurate quantitative information for particular populations of cells. Updated versions of this assay employ membranes coated with applied biological substrates allowing quantitative assessment of motility in response to substrate.

Another common method for the investigation of invasiveness is the 3-D matrix assay (for reviews, see Entschladen et al., 2005; Fischer et al., 1990), in which cells are embedded within a matrix composed of agarose or extracellular matrix components, and allowed to migrate through it. Included in this category is the under/over agarose assay in which a chemoattractant is placed into a hole cut into the agarose gel, and cells are placed either on top of the gel or in a separate hole and induced to migrate. These assays are more relevant to the *in vivo* condition, but are unsuitable for screening pharmacological inhibitors/stimulators due to the complexity of the experimental setup and the reliance on

diffusion properties of inhibitors and stimuli within the gel. Conversely, it is possible to take advantage of the slower diffusion to set up gradients for chemotactic investigation. The Dunn chemotaxis chamber (Zicha et al., 1991) is another 3D migration assay which is composed of concentric compartments allowed to communicate via a 1 mm bridge. When cells are placed in one compartment and a chemoattractant is placed in the other, this assay can provide real-time data on qualitative cellular responses toward stable linear concentration gradients. This assay is useful to study the motility of a wide variety of cell types, and the influence of a wide variety of chemoattractant substances, but does not allow for the investigation of the effects of alternate substrates on motility. Additionally, 3-D assays are often more difficult to analyze, and generally require time-lapse videography equipment for data collection and analysis.

The ability of the assay presented here to allow the study of motility over various applied substrates, while at the same time allowing the simultaneous study of quantitative and qualitative aspects of motility which can be influenced by a variety of stimulators, inhibitors and mutations, makes this assay a practical and viable alternative to existing assays, and may be useful to a wide variety of fields of study.



## **Chapter 6: A Laminin-Induced Upregulation of PTEN Promotes Migratory Behaviour in Cerebellar Granule Neurons**

*This study has been submitted for publication, 2008.*

### **6.0 Summary**

The development of proper cerebellar architecture is dependent upon the migration of cerebellar granule neurons (CGN) to the internal granule layer. The process of migration is, in turn, dependent on integrin receptors, which relay signals from the extracellular matrix (ECM) to the actin cytoskeleton. We have recently presented a novel signaling cascade whereby ECM component, laminin (LN), signals through integrin receptors to increase PTEN expression, which decreases p75NTR expression and subsequently depresses RhoA activity leading to enhanced axonal regeneration in PC12 cells. Since PTEN is required for cerebellar granule neuron migration, and a role for p75NTR in cerebellar foliation has recently been determined, the present study sought to determine if this ECM-induced signalling cascade is associated with the developmental migration of CGN. Western blotting shows that exposure to a LN substrate results in increased PTEN and decreased p75NTR expression in these cells over the first 8 days *in vitro* (DIV), a response that disappears after 14 DIV. This timeline of LN responsiveness correlates to the period of developmental migration *in vivo* and migratory behaviour *in vitro*. Furthermore, interference with the individual signalling components in this cascade impairs LN-induced motility *in vitro*. These results demonstrate a key role for ECM-induced upregulation of PTEN, and subsequent downregulation of p75NTR in CGN migration.

## 6.1 Introduction

The establishment of normal brain architecture in the developing nervous system depends upon the appropriate migration of neurons from their site of origin to their final destination in a specific lamina of the brain. The development of the cerebellum differs from cortex in that the migration of neurons occurs largely postnatally. Cerebellar granule neuron (CGN) precursors arise embryonically in the rhombic lip and migrate to form the external granule layer (EGL). After birth these precursors cease division, differentiate and migrate inwards through the molecular and the Purkinje layers, coming to reside in the internal granule layer (IGL). This migratory behaviour is guided by radial glial scaffolds (reviewed in Hatten, 1999; Komuro and Yacubova, 2003; Yacubova and Komuro, 2003).

The process of migration is often dependent on integrin signalling. The integrins are a group of integral membrane receptors that relay signals from the extracellular matrix (ECM) to the actin cytoskeleton to modulate cellular form and function. The formation of integrin-associated focal adhesions serves to anchor the leading process to the substrate and the subsequent turnover of focal adhesions is necessary to retract the trailing process. Furthermore, integrins are capable of initiating signalling cascades to change cellular behaviour. Binding of ECM components to their cognate receptor induces integrin clustering within the cell membrane forming focal aggregations which subsequently recruit a variety of kinases and adapter proteins to initiate signalling cascades that alter gene expression and thus manipulate cellular actions (reviewed in Moissoglu and Schwartz, 2006; Ridley et al., 2003).



We have recently presented evidence for a signalling cascade whereby a component of the ECM, laminin (LN), acts via integrin ligation to reduce the expression of p75NTR, the low affinity pan-neurotrophin receptor through the signalling intermediate PTEN, a phosphatase (Rankin et al. 2008b). p75NTR constitutively activates Rho, a small GTPase with known inhibition of actin cytoskeleton remodelling (Yamashita et al., 1999). The decrease in p75NTR expression results in sustained decrease of active Rho, thus eliminating the inhibition on lamellaepodial and filopodial extension thereby promoting growth in both NGF-differentiated PC12 cells (a sympathetic neuronal model) and in postnatal hippocampal neurons (Rankin et al. 2008b).

Each of these signalling components has been individually implicated in directing migratory behaviour of the developing cerebellum. LN deposits are associated with Bergmann radial glia (Liesi, 1992), and are known to promote growth and developmental migration of CGN (Powell et al., 1998). Additionally, PTEN has been implicated in proper migratory behaviour of CGN, as studies of PTEN-deficient mice demonstrate severely impaired lamina formation due to improper CGN migration (Backman et al., 2001; Groszer et al., 2001; Kwon et al., 2001; Marino et al., 2002; Nayeem et al., 2007). More recently p75NTR has been noted to play a role in the proper foliation of the cerebellum (Carter et al., 2003); a phenomenon often related to appropriate migration. Finally, Rho has a known role in cytoskeleton remodelling (Raftopoulou and Hall, 2004), and thus modulates the migratory behaviour of several cell types from *Dictyostelium* to fibroblasts (reviewed in Raftopoulou and Hall, 2004). Since cellular motility shares several of the same underlying processes as the extension of axonal outgrowth, we investigated whether this LN-initiated signalling cascade occurs in CGNs. Furthermore,

we sought to determine the outcome of cascade activation in this cell type. We report here that the LN-mediated PTEN-p75NTR signalling cascade does occur in CGN during a period of time corresponding to *in vivo* migration and that interference with the individual pathway components results in defective migratory behaviour *in vitro*.

## **6.2 Materials and Methods**

### **6.2.1 Primary Cell Culture**

Cultures enriched in granule neurons were obtained from cerebella of 8 day-old Sprague-Dawley rat pups as described previously (Jiang et al., 2003; Rankin et al., 2008). Cerebella were stripped of meninges, finely chopped and dissociated by enzymatic digestion using 0.25% trypsin-EDTA followed by mechanical trituration. The culture medium was Dulbecco's modified eagle medium (DMEM) supplemented with 10% FCS, 25 mM KCl and 1% penicillin/streptomycin solution (Invitrogen). Cells were seeded onto polylysine (PL; 40 µg/ml) or laminin (LN; 25 µg/ml)-coated tissue culture plates at a density of  $1.8 \times 10^6$  cells per ml. Cells were incubated at 37°C in 5% CO<sub>2</sub> and cytosine arabinoside was added to longterm cultures to inhibit the proliferation of non-neuronal constituents. On the seventh day in vitro (DIV) of longterm cultures, glucose (50 µl of a 100 mM solution) was added to each culture well to maintain survival, as culture medium was not replaced. This method yields cultures consisting of approximately 95% CGN (Jiang et al., 2003). For lentiviral transduction, cells were prepared with the following changes to the dissociation procedure, as dissociation using trypsin yielded no infection at the required timepoints. Briefly, in place of Trypsin-EDTA, neurons were enzymatically



digested with 5 ml papain (10U/ml) supplemented with EDTA, NaHCO<sub>3</sub> and DNase for 30 min at 36°C.

### **6.2.2 Western Blot Analysis**

For Western analyses, cells were harvested in the presence of sodium orthovanadate (100 mM in TBS) and subsequently subjected to lysis overnight at 4°C prior to centrifugation (10,000 x g, 5 min). A BCA protein assay (Pierce, Rockford IL) was used to determine protein concentration and equivalent amounts of protein (50 µg) were electrophoresed on 8% SDS-polyacrylamide gels. Protein was subsequently transferred to nitrocellulose membranes, which were then exposed to ponceau red to ensure equal protein loading. After washing in TBS, blots were blocked in 3% non-fat dry milk for 1h at RT, then incubated with primary antibody directed against PTEN (Cell Signalling), p75NTR (Upstate) or actin (Sigma) overnight at 4° C. A final incubation with HRP-conjugated secondary antibody (Chemicon) for 1h at RT was followed by visualization using an ECL method. Each experiment was repeated three times, subjected to densitometry and normalized to actin to ensure equal protein for comparison. Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc, San Diego CA) with significance being determined using one way ANOVA testing.

### **6.2.3 Real-time RT-PCR**

For real-time RT-PCR analyses, cells were subcultured on PL or LN-coated 12-well plates for varying timepoints as indicated. RNA was isolated using Trizol reagent (Invitrogen) as per the manufacturer's instructions, and was subsequently treated with DNase (Ambion, Austin TX) to remove traces of contaminating DNA. RNA (2 µg) was

reverse transcribed to cDNA using MMLV reverse transcriptase (200 U for 30 min at 37 °C; Invitrogen) prior to use as template for real-time PCR amplification using the following PCR primer pairs: p75NTR forward primer 5'-TGCATCTGAGCTGGTGTCTGTCTT and p75NTR reverse primer 5'-TGCGTACAATGCTCCTGGTCTCTT, PTEN forward 5'-ATTCGACTTAGACTTGACCT, PTEN reverse 5'-ACCAGTCCGTCCTTTC, 28s forward primer 5'-GACCAAGGAGTCTAACGC and 28s reverse primer 5'-GTACGCTCGTGCTCCA. PCR amplifications were performed using the Roche lightcycler and quantified using SYBR green I. p75NTR expression was subsequently normalized against 28s expression.

#### **6.2.4 Rho Activity Assay**

The activation of RhoA was assessed using a pulldown assay kit (Upstate) in accordance with the manufacturer's instructions. Briefly, cells were harvested and lysed. Rhotekin-bound beads were added to cleared lysates and rotated for 1h at 4° C prior to pelleting, washing and boiling in Laemmli buffer with DTT. Rhotekin-bound Rho proteins were detected by Western blot analysis using a polyclonal anti-Rho antibody (Upstate). The total amount of RhoA in cell lysates was used as a control for the cross-comparison of Rho activity.

#### **6.2.5 Motility Assay**

Motility was assessed using a previously described assay (Rankin et al., 2006). In brief, cells were plated in a monolayer on a PL-coated glass coverslip. Cells were allowed to attach and mature for the indicated time period before coverslips were inverted

onto 12-well tissue culture plates coated with PL or LN substrates. Cells were allowed to migrate from underneath the inverted coverslip into the migratory field for 36 h prior to image acquisition. Cell migration was quantified using a semi-automated cell counter plugin for Image J (NIH).

#### **6.2.6 Small Interfering RNA Constructs and Transfection**

PTEN siRNA was purchased from Cell Signalling (PTEN signal-silence siRNA). PTEN siRNA was introduced to the CGN using a calcium-phosphate co-precipitation method as previously described (Bingham et al., 2006; Rankin et al., 2008). Briefly, conditioned medium was removed and retained for replacement following transfection. Cells were washed twice with transfection medium (DMEM supplemented with 25 mM KCl). A calcium phosphate co-precipitate of siRNA oligos (100 nmol) was prepared and applied to the cells dropwise. Following an incubation of 2 h cells were washed twice with transfection media and returned to incubate in the retained conditioned medium. Cell analyses were performed 48 hours post transfection.

#### **6.2.7 Inhibitor Studies**

For pharmacological inhibition studies, cells were cultured on PL-coated coverslips. 2 h prior to establishment of motility assays, cells were primed with media supplemented with ROCK inhibitor Y27632, to a final concentration of 10  $\mu$ M. Coverslips were subsequently inverted into wells in the presence of the inhibitor and motility was assessed 36 hours later. Inhibitor concentration was determined empirically to provide the required inhibition without detriment to cell viability.



### 6.2.8 Lentiviral Constructs and Transduction

pcDNA3.1-p75NTR-Orange: Plasmid sequence encoding orange fluorescent protein was excised from the pRSET vector (a kind gift from Dr. Lois Mulligan) using BamHI and HindIII restriction enzymes and was inserted into the eukaryotic expression vector pcDNA3.1. Amplification of the full length p75NTR coding region was performed using forward primer 5'-CTCGAGATGAGGAGGGCAGGTGCTGC and reverse primer 5'-GATATCTGCACTGGGGATGTGGCAGTGG encoding XhoI and EcoRV restriction enzyme sequences which facilitated directional insertion into the engineered pcDNA3.1-orange vector yielding p75NTR with a C-terminus orange fluorescent tag. pGJ3-p75NTR-orange lentiviral construct: The third generation replication deficient lentiviral vector denoted pGJ3 was kindly provided by Dr. Michael Sendtner (University of Wurzburg, Germany). The p75NTR-orange fusion construct, including upstream CMV promoter and enhancer regions were excised from pcDNA3.1 and inserted into linearized pGJ3 lentiviral backbone. Lentiviral particles were produced by transfection of the 293T packaging cell line with the pGJ3-p75NTR-Orange construct, and an additional plasmid encoding VSV-glycoprotein to facilitate pseudotyping. 8 h post-transfection, viral transcription and packaging were induced by treatment with sodium butyrate (10 mM) for 8 h. Cell culture medium was collected at 24 h and 48 h post-induction, 0.45  $\mu$ m filtered to remove cellular debris, and ultracentrifuged for 90 min at 125,000 x g to concentrate lentiviral particles. Virus stocks were titred using 293HEK cells, and were frozen at -80° C until used for transduction of CGN at a multiplicity of infection (MOI) between 3 and 6.



## **6.3 Results**

### **6.3.1 LN-mediated regulation of PTEN and p75NTR occurs in cerebellar granule neurons**

Our previous study indicated that exposure to a LN substrate could induce an integrin-dependent upregulation of PTEN, a downregulation of p75NTR and a depression of active Rho which resulted in an enhanced regenerative growth response in NGF-differentiated PC12 cells and primary hippocampal neurons (Rankin et al. 2008b). In order to determine whether LN-induced changes in PTEN, P75NTR and Rho activity occur in CGN, dissociated CGN were obtained from 8 day old rat pups and plated on PL (40 µg/ml) or LN (25 µg/ml) and harvested at 24 h intervals for 8 DIV. Western blot analysis demonstrates that exposure to the LN substrate results in an upregulation of PTEN protein expression (Figure 6.1A), and a concomitant downregulation of p75NTR protein expression (Figure 6.1B) at each timepoint assessed.

LN-induced modulation of PTEN and p75NTR protein expression levels was confirmed by real-time RT-PCR analyses of PTEN and p75NTR mRNA expression (Figure 6.1C). Additionally, LN-induced changes in PTEN and p75NTR expression levels appear to be independent of neurotrophin stimulation with nerve growth factor (NGF; 50 ng/ml) or brain-derived neurotrophin factor (BDNF; 25 ng/ml) assessed after 8DIV (Figure 6.1D,E), although BDNF itself induces a well characterized upregulation of p75NTR in this cell type (Rankin et al., 2008). p75NTR is known to constitutively activate Rho (Yamashita et al., 1999). Since a LN-induced decrease in p75NTR was previously found to be associated with a decrease in active Rho (Rankin et al. 2008b),

**Figure 6.1: LN regulates PTEN- p75NTR signalling cascade in CGNs.** CGNs plated on PL or LN for 8 DIV were analyzed by Western blotting for PTEN (A) and p75NTR (B) expression. LN-induced upregulation of PTEN and downregulation of p75NTR was confirmed by real-time RT-PCR analyses (C). The influence of LN after 8DIV persisted regardless of neurotrophin stimulation with NGF or BDNF (D, E), and resulted in a depression in Rho activity as determined by a pull-down assay (F). Values expressed represent the mean expression relative to actin (A, B, D and E) or 28s (C) or total Rho (F) of three experiments +/- SEM where \* $p < 0.01$  as determined by one-way ANOVA (A-E) or T-Test (F). Values are expressed as a percentage of the control condition, PL.

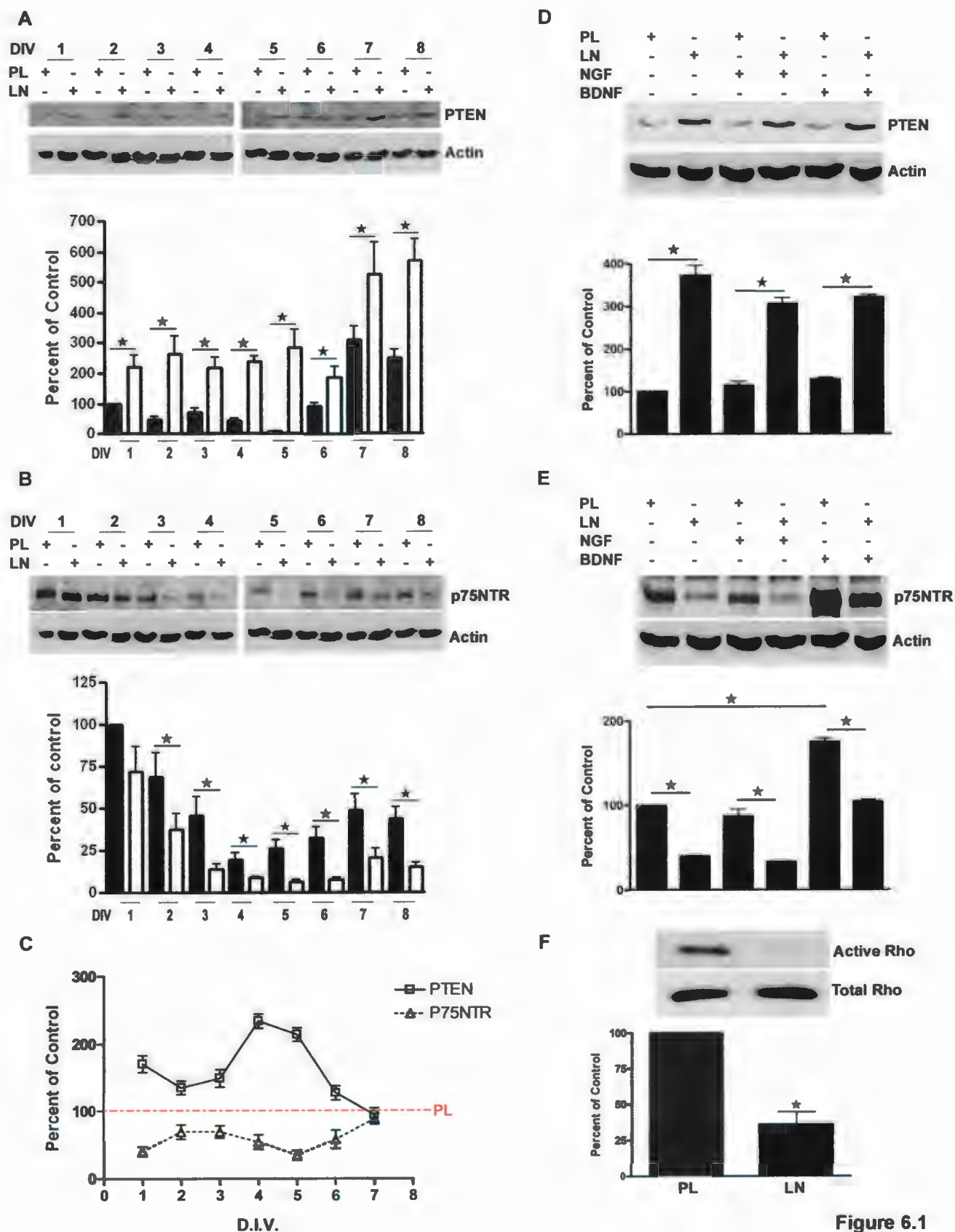


Figure 6.1

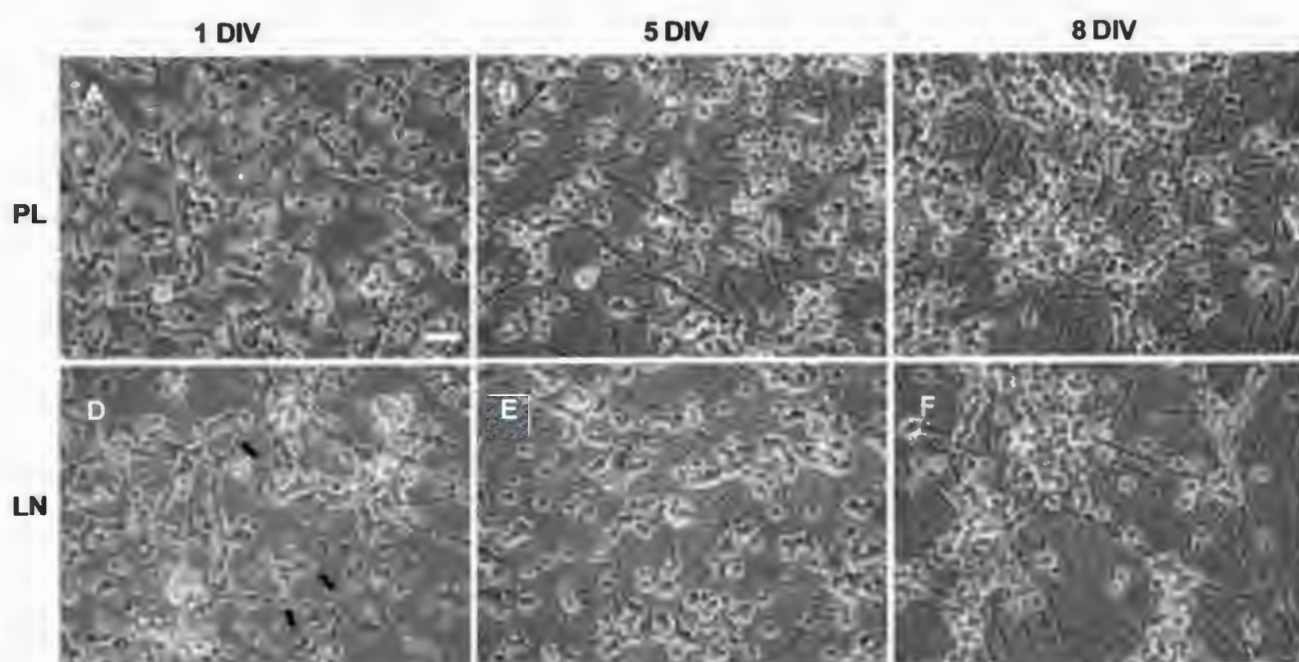
we assessed the effect of a LN substrate on Rho activity in CGN. Figure 6.1F shows decreased Rho activity in CGN plated on a LN substrate for 8DIV in comparison with those plated on PL. Thus, these data are consistent with the existence of a LN-induced PTEN-P75NTR-Rho signalling cascade in CGN.

### **6.3.2 Laminin induces early morphological changes**

This LN-induced signalling cascade was previously associated with a neurite growth response in PC12 cells and hippocampal neurons (Rankin et al. 2008b). LN is also well characterized to promote growth of early postnatal CGN (Powell et al., 1998). Thus, we analyzed CGN cultures plated on PL versus LN substrates for changes in cell growth over a period of 8 DIV. Comparison of phase contrast images present in Figure 6.2A and 6.2D suggests a modest enhancement of CGN growth when plated on a LN substrate (Figure 6.2D) in comparison with the PL control (Figure 6.2A). Slight differences in CGN growth observed at 24 h in culture are however indiscernable following 5 days in culture (Figure 6.2B and 6.2E), and CGN growth morphology is indistinguishable following 8 days in culture on either PL (Figure 6.2C) or LN (Figure 6.2F) substrates. Thus, while LN appears to enhance growth of CGN at an early timepoint post-plating, growth progresses normally on a PL substrate in the absence of integrin stimulation; differences in cell growth do not persist and thus appear incongruent with the observed differences in LN-induced PTEN and p75NTR expression observed in Figure 6.1. These results raised the possibility that this signalling paradigm may modulate other aspects of CGN behaviour in addition to neurite growth.



**Figure 6.2: Laminin induces early morphological changes which do not persist to 8 DIV.** Phase contrast micrographs of CGNs plated on PL (A-C) or LN (D-F) for 1 DIV (A,D), 5 DIV (B,E) or 8 DIV (C, F) show that early morphological differences are induced by the LN substrate at 1DIV, but are indistinguishable following culture for 5 or 8 DIV. Arrows indicate CGN that have taken on a typical elongated cell body and appear to have extended polarized processes. Scale bar = 25  $\mu\text{m}$ .



**Figure 6.2**

### 6.3.3 Cellular response to LN is temporally regulated and promotes migration

In addition to neurite growth, LN has also been reported to stimulate CGN migration. Since the bulk of CGN migratory activity occurs between postnatal days 7 – 12 (P7 – P12), the CGN isolated at P8 correspond to a highly motile population *in vivo*. Since migratory activity *in vivo* ceases by P21, we assessed the PTEN-p75NTR expression level changes in response to LN in CGN following 14 days cultured *in vitro*, corresponding to a timepoint by which all migratory behaviour has ended *in vivo* (P22). Western blot analysis demonstrates that CGN aged in culture for 14 days do not respond to a LN substrate by increasing PTEN (Figure 6.3A) or decreasing p75NTR (Figure 6.3B). The responsiveness of these cells to LN appears to correlate with the temporal pattern of migration observed *in vivo*. To test the migration of CGN *in vitro*, we employed a recently developed coverslip migration assay (Rankin et al., 2006) which facilitates exposure of the cells to a LN substrate at varying timepoints post isolation and allows the quantitative comparison of cell motility induced by various conditions. Analysis of CGN migration on PL or LN substrates commencing on DIV 1, 2, 3, 5, 7 or 10 reveals significantly greater cell motility upon LN exposure compared with PL (Figure 6.4A). However, migratory behaviour is absent, irrespective of substrate, by 14 and 22 DIV (Figure 6.4A). Following 1 DIV, CGN readily migrate in response to a LN substrate, while only a minor amount of non-neuronal migration is evident across the PL substrate (Figure 6.4B-D). CGN aged for up to 5 DIV prior to the initiation of the motility assay show a similar enhancement of motility on a LN substrate in comparison with the control PL condition (Figure 6.4E-G), while CGN cultured for 10 DIV prior to the initiation of the motility assay demonstrate minimal movement when plated on a LN



**Figure 6.3: CGNs response to LN is temporally regulated.** CGNs plated on PL or LN for 14 DIV were analyzed for PTEN (A) and p75NTR (B) protein expression, which did not significantly differ. PTEN and p75NTR expression values were normalized against actin and represent the mean of 3 experiments  $\pm$  SEM. Optical density values are expressed as a percentage of the control condition, PL.

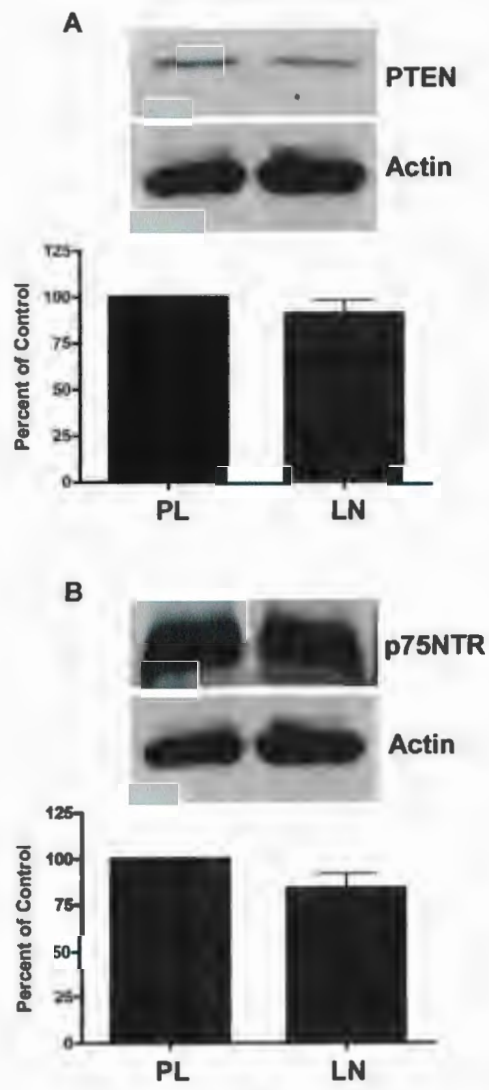
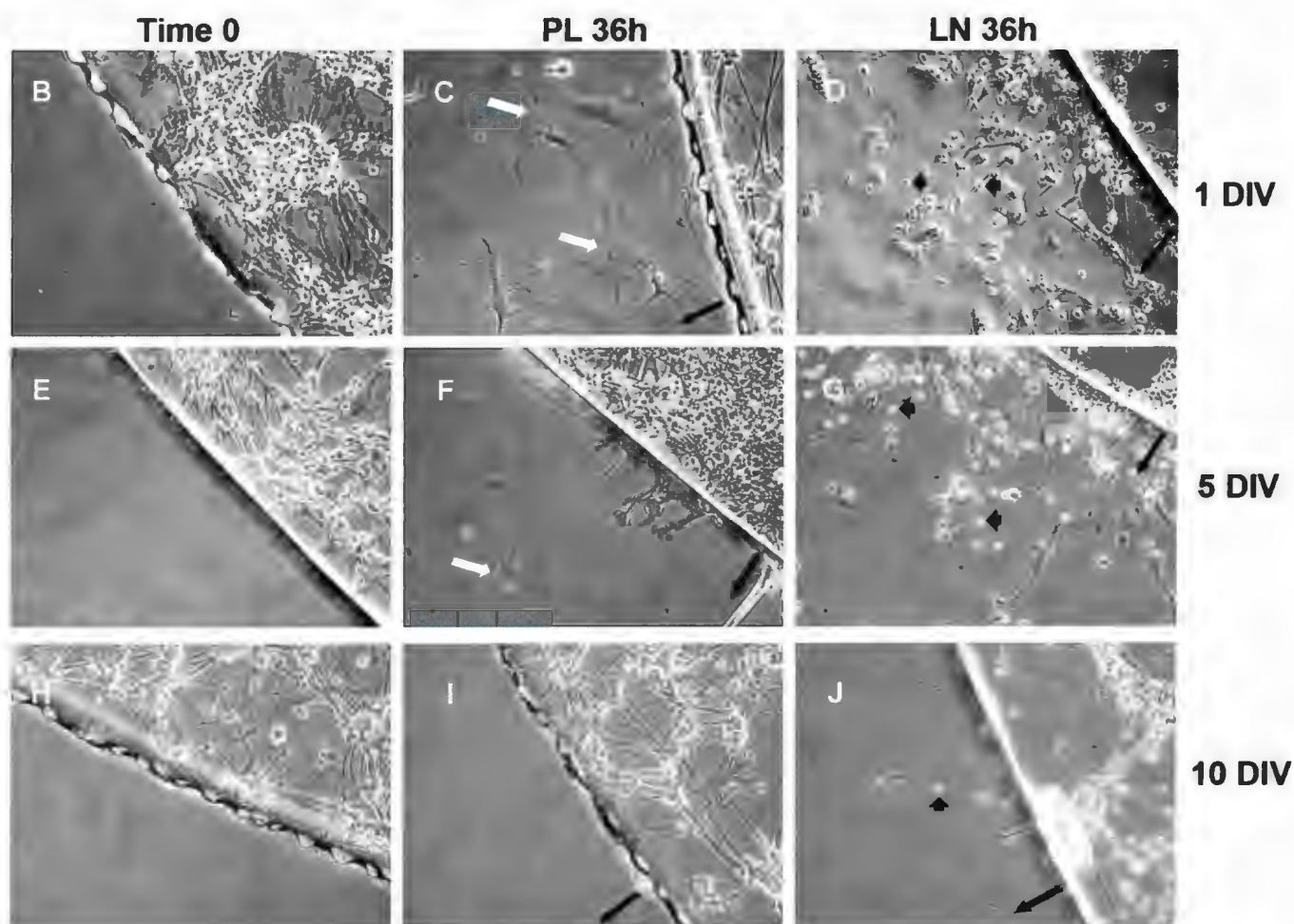
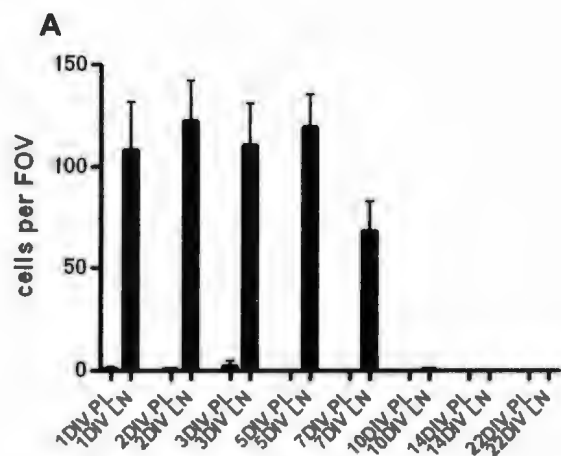


Figure 6.3

**Figure 6.4: The motile response of CGNs plated on a LN substrate is temporally regulated.**

The motile behaviour of CGNs was assessed using a coverslip assay wherein CGNs were plated on PL-coated coverslips and inverted over a PL or LN-coated migratory field following maturation in vitro for the time periods: 1, 2, 3, 5, 7, 10, 14, and 22 DIV, as indicated (A). Cell migration was assessed and quantitated following exposure to the migratory field for 36 h. Panels B – J are representative images of the cover slip assay initiated following 1, 5, or 10 DIV, across PL versus LN migratory substrates, as indicated. Long black arrows indicate the direction of movement, white arrows are indicating non-neuronal cells in the migratory field, and black arrowheads are indicating examples of CGN cell bodies. Motility was graphically represented in (A) following enumeration of motile cells in 40 fields of view (FOV) per coverslip, with 3 coverslips imaged per condition in a total of 3 independent experiments.





**Figure 6.4**

substrate and a complete absence of motility in response to PL (Figure 6.4H-J). These results strongly suggest that LN responsiveness in terms of both altered PTEN-p75NTR expression and subsequent motile behaviour corresponds to the developmental migration of CGN *in vivo*.

#### **6.3.4 Perturbation of individual signalling components of the cascade inhibits migration**

To confirm that LN-induced changes in PTEN and p75NTR are responsible for modulation of CGN motility *in vitro*, we assessed the ability of the individual signalling components to modify the cellular response to LN. Since LN induces an upregulation of PTEN, we evaluated whether RNAi-mediated knockdown of PTEN expression would prevent cell motility in response to a LN substrate. As shown in Figure 6.5A-B, a LN substrate readily induces migration of CGN transfected with a fluorescently tagged scrambled siRNA sequence. In contrast, CGN treated with PTEN-specific siRNA demonstrate significantly impaired motility on a LN substrate (Figure 6.5C-D). Graphical representation of CGN motility following control scrambled or PTEN siRNA is depicted in Figure 6.5E, and Figure 6.5F illustrates the effective PTEN protein downregulation at 48 h by the siRNA delivery to the CGN.

PTEN upregulation results in a decrease of p75NTR (Rankin et al. 2008b). To determine if this association is necessary for appropriate migratory behaviour, we overexpressed p75NTR in CGN using lentiviral constructs and subsequently exposed them to a LN substrate for 36 h prior to quantitation of the motility of fluorescent cells. Relative to the empty vector control (Figure 6.6A-B), p75NTR

**Figure 6.5: PTEN siRNA impairs motility of CGNs on a LN substrate.** CGNs were plated on PL coated coverslips and transfected with fluorescently-labeled scrambled (A,B) or unlabeled PTEN siRNA (C,D) prior to inversion on a LN substrate. Motility was imaged at time 0 (A,C) or 36 h post-inversion (B,D). Arrows indicate the direction of movement. (E) represents graphical quantitation of motile behaviour, determined by enumeration of cells migrating from underneath the coverslip. Motility is impaired by transfection with PTEN siRNA relative to the scrambled control. PTEN siRNA significantly reduces PTEN protein expression, as determined by optical density and expressed as a percent of the control siRNA condition (F). Values represent quantitation of 40 brightfield images per coverlip, with 3 coverslips imaged per condition in a total of 3 independent experiments. \* $p < 0.0001$ .



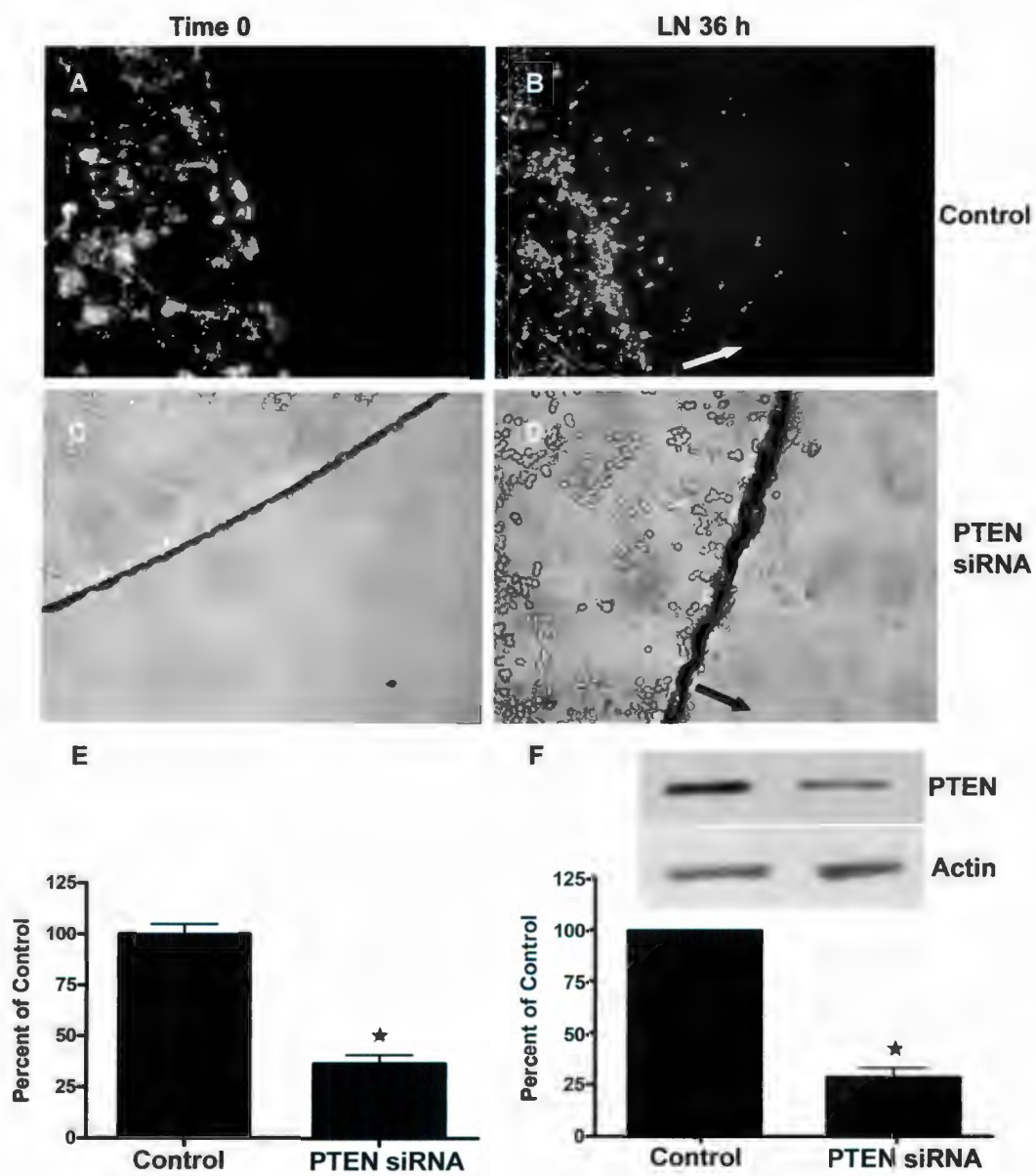


Figure 6.5

**Figure 6.6: p75NTR overexpression impairs motility of CGNs on a LN substrate.** CGNs were plated on PL coated coverslips and transduced with lentiviruses encoding orange fluorescence (A,B) or fluorescently-tagged p75NTR (C,D) prior to inversion onto a LN substrate. Motility was imaged at time 0 (A,C) or 36 h post-inversion (B,D). Arrows indicate the direction of cell movement. CGNs transduced with lentiviruses encoding the p75NTR fusion construct (F) show reduced motile behaviour to the LN substrate (E) as determined by enumeration of cells migrating from underneath the coverslip. Quantitation involved 40 FOV per coverslip, with 3 coverslips imaged per condition in a total of 3 independent experiments. \* $p < 0.0001$ .

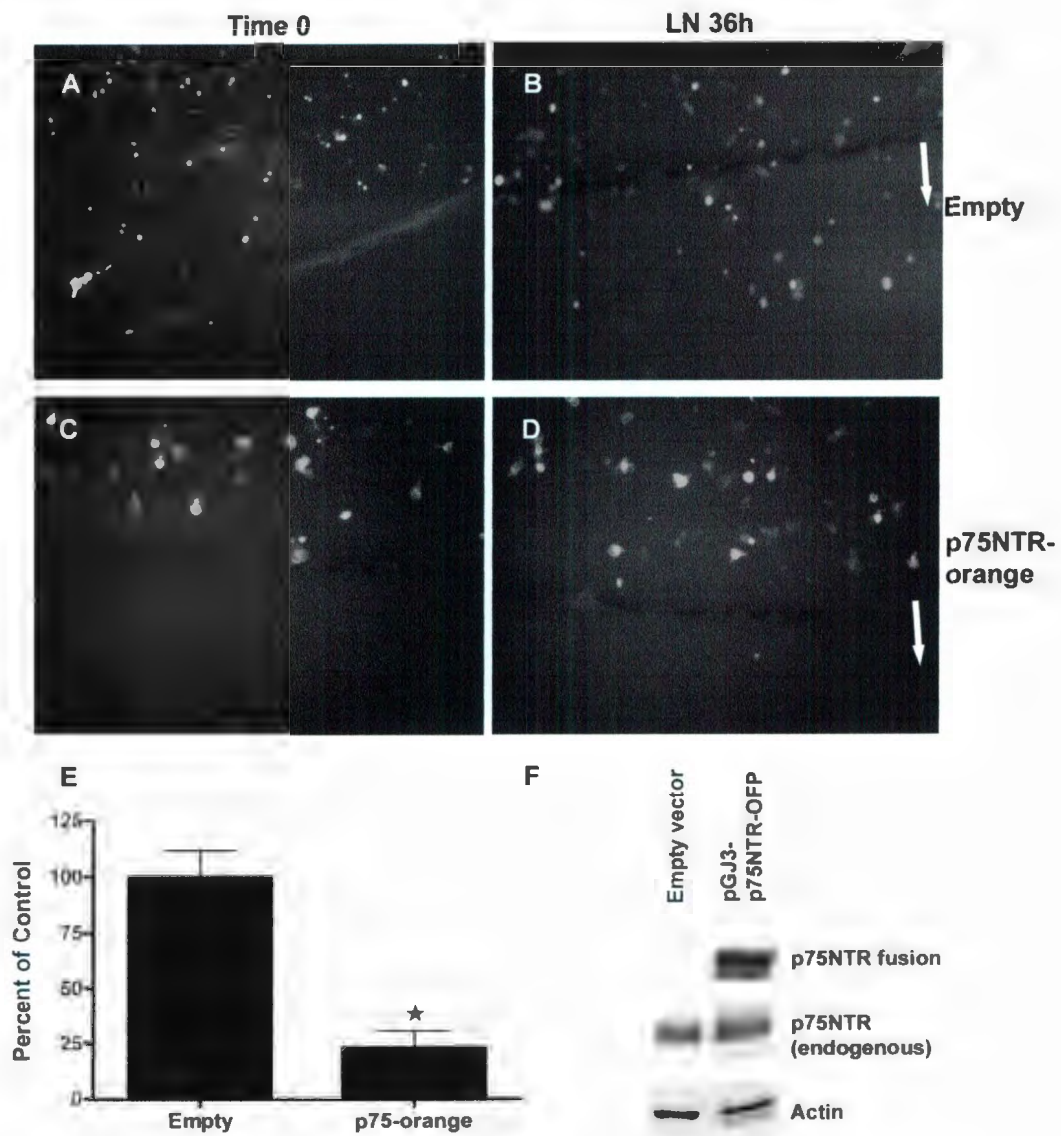


Figure 6.6



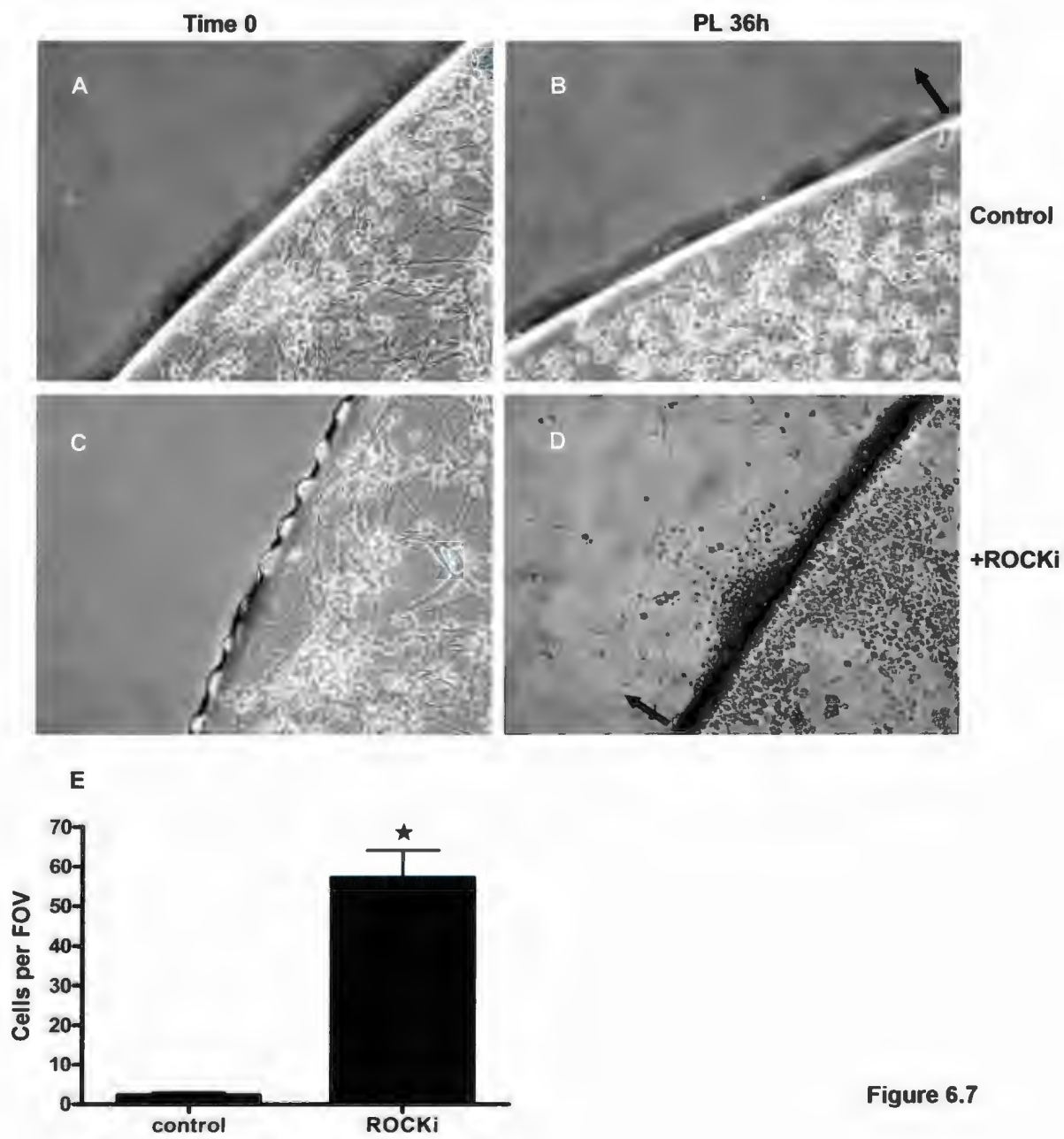
overexpression impairs the LN-induced motility of CGN (Figure 6.6C-D). This impairment is significant, as depicted by graphical representation in Figure 6.6E.

Finally, to assess whether the decrease in Rho activity observed on a LN substrate promotes motile behaviour, we utilized a pharmacological inhibitor of a downstream Rho effector molecule, Rho kinase (ROCKi; Y27632, 10 $\mu$ M). There is a clear absence of motile behaviour on the PL substrate in the absence of the ROCKi (Figure 6.7A,B). Inclusion of this inhibitor in the cell culture medium facilitates the motility of CGN across a PL substrate (Figure 6.7C-D), as quantitatively determined and presented in Figure 6.7E. Since manipulation of each component of this PTEN-p75NTR-Rho signalling cascade can alter the CGN motile response, these results suggest that this pathway is important for regulation of CGN motile behaviour in response to LN.

In order to confirm the hierarchy of this signalling cascade in the regulation of cell motility, we assessed whether or not inhibition of downstream Rho activity using ROCKi could rescue the motility impairments induced by PTEN siRNA and p75NTR overexpression. P8 CGN were isolated, plated on PL-coated coverslips, and transfected with either control siRNA or PTEN siRNA. 48 h post-transfection, coverslips were inverted onto LN-coated wells containing medium supplemented with Rocki Y27632. Following an additional 36 h, migration was assessed. As shown in Figure 6.8A-E, the decrease in motility caused by PTEN siRNA can be restored to levels not significantly different from control by the downstream inhibition of Rho kinase. Additionally, P8 CGN were isolated, plated onto PL-coated coverslips and infected with lentiviruses carrying sequences for either orange fluorescent protein (empty control) or p75NTR-orange, and inverted into LN-coated wells containing medium supplemented with Rocki

**Figure 6.7: Inhibition of Rho kinase promotes CGN motility on a PL substrate.** CGNs were plated on PL coated coverslips and inverted into cell culture media in the absence (A, B) or presence (C, D) of the ROCK inhibitor, Y27632. Motility was imaged at time 0 (A, C) or 36 h post-inversion (B, D). Black arrows indicate the direction of cell movement. Treatment with ROCKi induces motile behaviour in CGN (E) as determined following quantitation of 40 images per coverlip, with 3 coverslips imaged per condition in a total of 3 independent experiments.

\* $p < 0.0001$ .



**Figure 6.7**

**Figure 6.8: Inhibition of Rho kinase can restore motility impairments induced by PTEN siRNA or p75NTR overexpression.** CGNs were plated on PL-coated coverslips and transfected with PTEN siRNA (A-E) or transduced with lentiviruses encoding fluorescently tagged p75NTR (F-J). Motility was assessed following inversion onto a LN substrate for 36 h in the presence or absence of ROCK inhibitor Y27632, and is graphically represented in (A) and (F) following quantitation of 40 fields of view per coverslip, with 3 coverslips imaged per condition in a total of 3 independent experiments. Representative images of PTEN siRNA-transfected (B-E) or p75NTR-transduced (G-J) CGN are depicted. Arrows indicate the direction of cell movement. \* $p < 0.05$ , \*\* $p < 0.001$ .



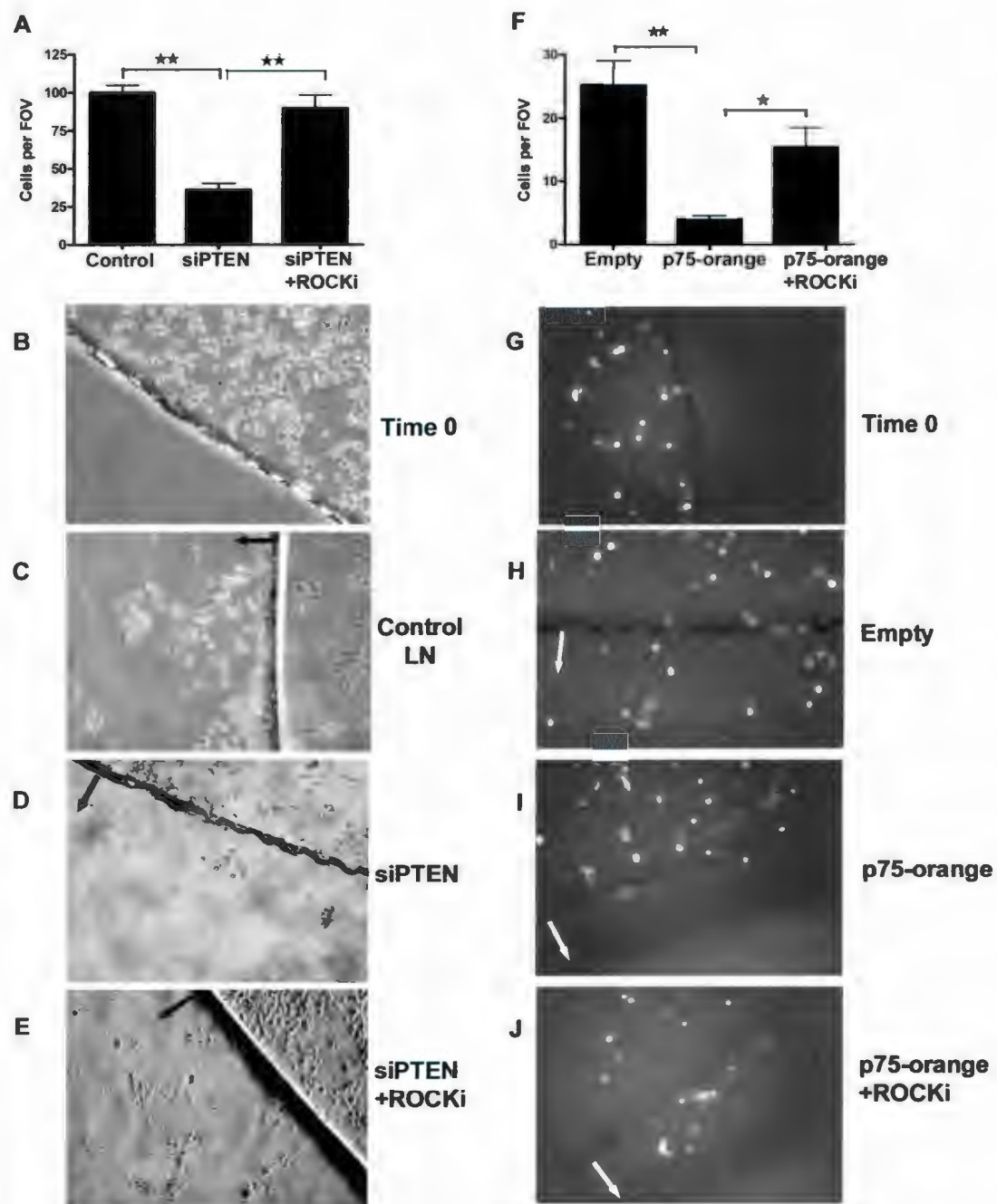


Figure 6.8

Y27632 after 48 h. Following an additional 36 h, migration was assessed. As shown in Figure 6.8F-J, the decrease in motility caused by p75NTR overexpression can be partially restored by the downstream inhibition of Rho kinase. These results confirm the involvement of these signalling components in the regulation of CGN motility, and confirm that Rho and Rho kinase are downstream components in this signalling cascade.

#### **6.4 Discussion**

The development of appropriate cerebellar architectonics is in large part reliant upon the process of directed cell migration, which in turn, relies upon the integration of a multitude of extracellular cues (short range, long range, soluble and substrate-bound) derived from the physiological environment in which cells reside (Hatten, 1999). These migration cues are tightly regulated in terms of temporal and spatial expression throughout development, thus accurately guiding granule cell precursors from their origin in the rhombic lip to the subpial proliferative zone, EGL, and following differentiation, to their ultimate residence in the IGL (Komuro and Yacubova, 2003; Yacubova and Komuro, 2003). External cues are essential for the selection of an appropriate migratory pathway as well as the termination of cell movement (Hatten, 1999), but the molecular mechanisms influencing the post-mitotic migratory behaviour of CGN are not well understood.

In this study, we have used an *in vitro* CGN culture paradigm to determine if an ECM-induced signalling cascade previously shown to promote growth in CNS neurons could play a role in the developmental migration of post-mitotic CGNs. CGN isolated from 8 day old rat pups were cultured on PL versus LN-coated substrates to investigate

LN-mediated changes in PTEN, p75NTR and Rho, and neurons of varying ages were exposed to LN to discern the temporal regulation of this signal transduction and the subsequent migratory behaviour. We demonstrate that LN induces an upregulation of PTEN and a downregulation of p75NTR protein expression in CGN, resulting in a decrease in active Rho and promotes cellular motility in an *in vitro* assay. *In vitro* studies of CGN migration have previously been reported to correlate well with their *in vivo* counterparts, in terms of time scale, speed, dynamics and basic patterning (Tarnok et al., 2005) demonstrating that the intrinsic characteristics of migration can be preserved *in vitro*, though the stereotypical unidirectional migration (i.e. away from EGL only) is lost (Komuro and Rakic, 1995).

In rats, the migration of post-mitotic granule cell neurons is most active during the first 2 postnatal weeks with maximum migration occurring between postnatal days 7 and 12. The EGL is no longer histologically evident by P15 and all migration is thought to be complete by P21 (Liesi, 1985; Yue et al., 2005). By enzymatically dissecting CGN from P8 animals, we are effectively isolating EGL CGN, some of which have begun to migrate (Raetzman and Siegel, 1999), a behaviour we can encourage by exposure to the LN substrate, but not a PL substrate.

LN has long been known to promote both *in vitro* growth (Powell et al., 1998) and the rapid and frequent migration of CGN (Fishell and Hatten, 1991; Fishell et al., 1993; Liesi, 1990) in a manner that is dependent on integrin ligation but which is independent of the presence of glial cell processes (Liesi, 1992). This migration can be blocked by antibodies to the growth promoting domain of LN (Liesi et al., 1992; Selak et al., 1985)



or by genetic deletion of integrin  $\beta 1$  in neurons and glia (Graus-Porta et al., 2001), both of which result in ectopic localizations of CGN and poor cerebellar foliation.

The spatial and temporal regulation and molecular distribution of LN corresponds to a role in both growth and migration, as these processes are intertwined in the cerebellum; axon extension must precede cell body migration (Liesi, 1985; Sobeih and Corfas, 2002). LN is a component of the basement membrane, and is well characterized to direct migration of granule cell precursors from the rhombic lip (Luckenbill-Edds, 1997). LN is also localized to sites distinct from the basement membrane early in development and has been reported to associate with the EGL granule cell precursors and form punctate deposits along the Bergman radial glia whose processes form scaffolds for pioneering neurites (Liesi, 1992). In the CNS, LN expression does not persist to adulthood (Powell et al., 1998). In fact, by P24 all LN immunoreactivity is lost from Bergman radial glia, EGL and Purkinje cells. This corresponds to a timepoint when all migration is complete (Liesi, 1985). We detected no migration after 10 DIV (analogous to P18) in our *in vitro* assay, indicating that the cells intrinsically lose the ability to respond to LN, thus indicating changes in the signal transduction pathways of the cell.

One of these intrinsic changes was the ability of LN to induce PTEN upregulation, which was lost with age *in vitro*. PTEN is a dual specificity phosphatase that has been previously noted to play a role in migration, in a variety of cell types (Leslie et al., 2005). In general, PTEN expression is inhibitory to migration, and is noted to suppress the migratory behaviours of mouse embryonic fibroblasts, B-lymphocytes, tumour cells and *Dictyostelium* (Liliental et al., 2000; Lim et al., 2004; Suzuki et al., 2003) using either or both of its lipid and protein phosphatase activities (Gao et al., 2005; Leslie et al., 2007;



Liliental et al., 2000; Yamada and Araki, 2001), or simply its C2 domain in a cell type-specific fashion (Raftopoulou et al., 2004). Proposed mechanisms include its impact on PDK-1-Akt signalling (Lim et al., 2004), dephosphorylating Fak and Shc to prevent focal contact formation and p130Cas signalling (Gu et al., 1998; Tamura et al., 1998), or simply antagonizing the ability of PI3K to promote migration. Nonetheless, a different role emerged from the genetic deletion of PTEN in the cerebellum. Global PTEN knockout extensively impairs embryogenesis resulting in embryonic death precluding the study of cerebellar formation. However, selective knockout of PTEN in specific tissues and cell types has allowed the study of PTEN in the development of the cerebellum. All PTEN knockout animals which selectively inactivate PTEN in neural precursors (e.g. utilizing Nestin or Engrailed2 promoters) display severely impaired CGN migration and positioning leading to distorted foliation, ectopia and substantial architectural abnormalities indicating a role for PTEN in positively regulating migration in this cell type (Backman et al., 2001; Groszer et al., 2001; Kwon et al., 2001; Marino et al., 2002; Nayeem et al., 2007). One study selectively eliminated PTEN using the hGFAP promoter, and attributed lack of oriented migration to defects in the differentiation of Bergman radial glia and therefore loss of appropriate guidance (Yue et al., 2005), highlighting another potentially important role for PTEN in cerebellar formation.

We have previously demonstrated that one downstream effect of PTEN expression is downregulation of p75NTR (Rankin et al. 2008b), which has a widespread role in development of the nervous system, both by modulating the growth response and controlling developmental apoptosis which regulates innervation density (Roux and Barker, 2002). The role of p75NTR in migration is understudied, but its temporal and

spatial regulation in the cerebellum is consistent with a role in development (Carter et al., 2003), and a role in cerebellar patterning and foliation has been proposed.

p75NTR knockout mice show no defects in foliation (Carter et al., 2003), a result that is consistent with our hypothesis that downregulation of this receptor promotes appropriate migration. In fact, we demonstrate that p75NTR overexpression impairs the migratory response to LN. The impact of p75NTR on migration could be due to loss of its downstream signalling capabilities, as ceramide, a product of p75NTR-mediated sphingomyelinase activation, has recently been shown to play a role in plasma membrane reorganization and thus migration (Falluel-Morel et al., 2005). Alternatively, the impact of p75NTR on migration is also likely related to the ability of p75NTR to constitutively activate Rho, a small GTPase known to regulate dynamics of the actin cytoskeleton thereby potentially inhibiting neurite outgrowth (Yamashita et al., 1999).

Rho activity in migrating cells is associated with focal adhesion assembly and the formation of stress fibres by activating downstream effectors which enhance net polymerization of actin and crosslink it, thus increasing contractility (Ridley et al., 2003). This is converse to Rac and cdc42 which regulate leading edge protrusion. In our studies, the LN substrate is associated with a decreased level of active Rho, a phenomenon previously reported for fibronectin, another ECM component (Ren et al., 1999; Ren et al., 2000). The ECM acts via integrins to activate FAK, which is reported to suppress Rho activity leading to focal adhesion turnover, a necessary element of migration. We further demonstrate that inhibition of downstream Rho signalling can restore the migratory defects imposed by loss of PTEN or overexpression of p75NTR, confirming that Rho signalling is downstream to the PTEN and p75NTR changes in this migratory paradigm.

The appropriate migration of cells is imperative to nervous system development, but is also essential throughout life during tissue regeneration, wound healing and in facilitating immunity. The extracellular cues that direct migration cascades and mediate transcriptional changes necessary for cytoskeletal organization for movement have been previously independently linked to migration of CGN. This signalling cascade cohesively links the ECM, PTEN, p75NTR and Rho in a single mechanism to elicit a biological reaction necessary for development.



## **Chapter 7: PTEN downregulates p75NTR expression by decreasing the DNA binding activity of Sp1**

*This study has been accepted for publication in Biochemical and Biophysical Research Reports, December 2008, In press.*

### **7.0 Summary**

p75NTR is broadly expressed throughout the nervous system and its regulation is essential for the growth, development and survival of neurons. The dysregulation of p75NTR is associated with a multitude of pathological conditions. It is thus of broad interest to elucidate mechanisms underlying the regulation of p75NTR expression. We have recently demonstrated a signalling cascade initiated by ECM molecule laminin (LN), which upregulates PTEN and downregulates p75NTR. Here we investigate the mechanism by which PTEN modulates p75NTR. Studies using PTEN mutants show that its protein phosphatase activity directly modulates p75NTR. Nuclear relocalization of PTEN subsequent to LN stimulation suggests transcriptional control of p75NTR expression, which was confirmed following EMSA and ChIP analysis of Sp1 transcription factor binding activity to the p75NTR promoter. LN and PTEN each independently decrease the DNA binding ability of Sp1 to its consensus sequence and the p75NTR promoter. PTEN regulation of p75NTR occurs via dephosphorylation of Sp1, thus reducing p75NTR transcription and protein expression. This mechanism represents a novel regulatory pathway which controls the expression level of a receptor that has broad implications not only for the development of the nervous system but also for progression of pathological conditions.



## 7.1 Introduction

p75NTR is a pleiotropic receptor best known for its role as a low-affinity pan-neurotrophin receptor working co-ordinately with the Trk family of receptor tyrosine kinases, transducing the signals from NGF, BDNF, NT3 and NT4/5 to regulate a broad array of processes essential to the development and maintenance of the nervous system (Blochl and Blochl, 2007). There is no intrinsic enzymatic activity associated with the cytoplasmic domain of p75NTR. Instead, the physiological outcome of activation is dependent upon the availability of different co-receptors and the selective recruitment of intracellular signalling partners (Roux and Barker, 2002).

In addition to functionally collaborating with Trk to enhance growth, p75NTR also initiates a variety of Trk-independent effects, which are largely detrimental to cell survival and growth. p75NTR constitutively activates Rho, a small GTPase known to potentially inhibit actin remodelling for growth, a relationship which is negated upon ligand binding (Yamashita et al., 1999). p75NTR also plays a central role in the transduction of myelin-derived signals which are inhibitory to growth (Wang et al., 2002a). Furthermore, p75NTR ligation results in activation of several apoptotic cascades (Rabizadeh and Bredesen, 2003). The dysregulation of p75NTR expression is central to a variety of pathological processes, as well. For example, p75NTR is implicated as a receptor for rabies virus (Langevin et al., 2002) and soluble  $\beta$ -amyloid (Sotthibundhu et al., 2008), is a marker for invasive metastatic glioma (Johnston et al., 2007), and has been implicated in the pathogenesis of multiple neurodegenerative disorders (Dechant and Barde, 2002; Schor, 2005). The mechanism of p75NTR regulation is therefore of broad importance.

We have recently demonstrated evidence for a signalling cascade by which laminin (LN), an extracellular matrix (ECM) component, can regulate p75<sup>NTR</sup> expression to promote growth and motility (Rankin et al. 2008b). This ECM-mediated signal transduction is associated with an upregulation of PTEN, a dual specificity phosphatase best known as a tumor suppressor. In this study, we have investigated the mechanism by which PTEN results in the transcriptional downregulation of p75<sup>NTR</sup>.

## **7.2 Materials and Methods**

### **7.2.1 Cell Culture and Differentiation**

The experimental model consisted of rat pheochromocytoma cells (PC12) (gift from Dr. David Kaplan, Hospital for Sick Children, Toronto ON) which were maintained on rat-tail collagen-coated tissue-culture flasks in RPMI-1640 medium (Invitrogen) supplemented with 10% horse serum, 5% fetal calf serum and 1% penicillin/streptomycin/glutamine solution (Invitrogen). Cells were incubated at 37 C in 5% CO<sub>2</sub> and cultured to 80% confluence prior to trypsinization for subculturing and differentiation. Cells were differentiated by exposure to NGF (100 ng/ml) for 2 days in low serum conditions to prime, followed to NGF (100 ng/ml) for 5 days in the absence of serum.

### **7.2.2 Primary Neuronal Culture**

Cultures enriched in granule neurons were obtained from cerebella taken from 8d old Sprague-Dawley rat pups as previously described. Cerebella were stripped of



meninges, finely chopped and dissociated using enzymatic digestion using 0.5% trypsin-EDTA followed by mechanical trituration. The culture medium was Dulbecco's modified eagle medium (DMEM) supplemented with 10% FCS, 25 mM KCl and 1% penicillin/streptomycin solution (Invitrogen). Cells were seeded onto polylysine (PL) (40 µg/ml) or laminin (LN)-coated (25 µg/ml) 12-well tissue culture plates of  $1.8 \times 10^8$  cells/ml. Cells were incubated at 37 C in 5% CO<sub>2</sub> and cytosine arabinoside was added 24 h later to inhibit proliferation of non-neuronal constituents. On the seventh day in vitro (DIV) glucose (50 µl of a 100 mM solution) was added to each culture well to maintain survival as culture medium was not replaced. Cultures were used on 8 DIV unless otherwise specified. This method yields cultures consisting of approximately 95% cerebellar granule neurons (Jiang et al, 2003).

### **7.2.3 PTEN Plasmid Constructs and Transfection**

pIRES-PTEN: full length rat coding sequence was amplified from cDNA using PCR forward primer 5'-GAATTC CATGACAGCCATCATC and reverse primer 5'-GGATCCTCAGACTTTTGTAATTTGTG incorporating EcoRI and BamHI restriction enzyme sequences respectively. The resulting PCR product was cloned into the TOPO TA cloning vector 2.1 (Invitrogen), subsequently excised using EcoRI and BamHI and directionally inserted into the bicistronic reporter vector pIRES (Promega). Empty pIRES control or PTEN-pIRES vectors were transfected into differentiated PC12 cells using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. GFP-tagged wild-type or C124S and G129E mutant PTEN constructs were a kind gift from Dr.N.R.Leslie (University of Dundee, UK) and were transfected as above.

#### **7.2.4 Western Blot Analysis**

For Western blot analyses cells were harvested in the presence of sodium orthovanadate (100mM in TBS) and subsequently subjected to lysis overnight at 4 C prior to centrifugation (10,000 x g, 5 min). A BCA protein assay (Pierce Rockford IL) was used to determine protein concentration and equivalent amounts of protein (50 µg) were electrophoresed on 8% SDS-polyacrylamide gels. Protein was subsequently transferred to nitrocellulose membranes that were then exposed to ponceau red to ensure equal protein loading. After washing in TBS, blocks were blocked in 3% non-fat dry milk for 1 h at room temperature prior to incubation with primary antibody overnight at 4 C. A final incubation with HRP-conjugated secondary antibody (Chemicon, Temecula CA) for 1 h at RT was followed using visualization using an ECL method. Each experiment was repeated three times, subjected to densitometry and normalized to actin to ensure equal protein for comparison. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software Inc, San Diego CA) with significance being determined using one way ANOVA testing or student's t test as appropriate.

#### **7.2.5 Immunocytochemistry**

For immunocytochemical studies cells were trypsinized and subcultured serum-free on PL or LN-coated 16-well chamber slides for 24h in the presence or absence of NGF (50ng/ml) prior to fixation in 4% paraformaldehyde for 15 min. Cells were then permeabilized with 0.1% TritonX-100 and blocked with 10% normal goat serum in PBS for 1 h at RT. Cells were incubated with a monoclonal antibody directed against PTEN (Cell Signaling) for 16 h at 4 C followed by incubation with Cy5-tagged secondary



antibody (Jackson ImmunoResearch Laboratories Inc) for 1h at RT. Slides were coverslipped with glycerol and expression patterns were visualized using confocal laser scanning microscopy.

#### **7.2.6 Protein Fractionation**

Cells were subcultured on PL or LN-coated tissue culture plates and protein was collected using the NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce) as per manufacturer's directions.

#### **7.2.7 Electrophoretic Mobility Shift Assay**

After exposure to PL or LN substrate, cells were washed in cold TBS, collected and fractionated as described above. Oligonucleotides complementary to the SP1 binding sequence, as well as oligonucleotides comprising a mutated SP1 consensus sequence (Ramos et al, 2007) were synthesized by Integrated DNA Technologies.

Oligonucleotides were annealed using 10 pmol of each probe in a 10  $\mu$ l reaction containing 20 mM Tris-HCL (pH 8.4) and 50 mM KCl which was incubated at 80 C for 5 min, followed by cooling to RT. The Sp1 binding region of the rat p75NTR promoter was amplified by PCR using forward primer 5'-ACAGAGAAGCCGCAGCGG and reverse primer 5'GGCGCTGCAGGCAGCACC, and were subsequently cloned using a TOPO TA cloning kit (Invitrogen). The p75NTR promoter region was excised using vector-derived flanking restriction enzymes, electrophoresed using 1% agarose, and purified using a gel extraction kit (Qiagen). Double-stranded DNA oligos were end-labeled using T4 polynucleotide kinase and  $\gamma^{32}$ -dATP, while the purified p75NTR promoter region was radiolabelled using large fragment of DNA polymerase (Klenow)

and  $\gamma^{32}$ -dATP. Radiolabelled probes were precipitated with 0.1 vol 3M NaAc and 3 vol 100% ethanol at -20 C for 1 h and pelleted by centrifugation at 12,000 x g for 20 min at 4 C. DNA pellets were washed with 70% ethanol, centrifuged for 10 min and resuspended in 30  $\mu$ l H<sub>2</sub>O. 15,000 cpm were incubated for 25 min on ice with 10  $\mu$ g nuclear extract in the presence of 20 mM Tris-HCL (pH 7.5), 75 mM KCl, 3.5 mM DTT, 20 nM ZnCl<sub>2</sub>, 1  $\mu$ g/ $\mu$ l BSA, 5% glycerol and 1  $\mu$ g poly(dI-dC) in a total volume of 20  $\mu$ l. To confirm specificity of DNA binding, unlabelled competitor DNA (wt or mutant sequence) was added to the binding in 100-fold excess of the hot probe and allowed to incubate for 20 min on ice. Following incubation with cold probe, hot probe was added and samples incubated for a further 30 min on ice. Samples were electrophoresed on a 5% non-denaturing polyacrylamide gel in TBE (89 mM Tris, 89mM boric acid, 2 mM EDTA) for 1.5 hr at 120V for Sp1 binding assays, or 3.5 hr at 120V for p75NTR promoter binding assays. Gels were subsequently dried under vacuum at 80 C for 2 h and visualized by autoradiography.

### **7.2.8 Chromatin Immunoprecipitation**

Chromatin immunoprecipitations (ChIP) were performed using the chromatin immunoprecipitation assay kit (Millipore) according to manufacturer's instruction. In brief,  $1 \times 10^6$  cells were crosslinked by addition of 0.8% formaldehyde directly to the cell culture media for 10 min at 37 C and subsequently quenched with 0.125 M glycine for 5 min at RT. Cells were washed twice with cold PBS, collected and pelleted, and finally resuspended in 200  $\mu$ l SDS lysis buffer. Chromatin was then sonicated to an average length of 0.5 kb by 3 x 20 sec pulses at 15 % power. Lysate was centrifuged at 14,000



rpm for 10 min at 4 C, diluted 10X in dilution buffer and precleared for 30 min at 4 C with 80 µl salmon sperm DNA/protein A agarose. 100 µl of diluted supernatant was retained for isolation of input DNA and to quantitate the DNA in different samples. Immunoprecipitation was performed using 4 µg anti-Sp1 or control IgG at 4 C overnight with rotation. 60 µl of salmon sperm DNA/protein A agarose was added for 1h at 4 C with rotation to collect the antibody-antigen-DNA complex. Complexes were then washed with low salt washing buffer (20 mM Tris, 2 mM EDTA, 0.1% SDS, 1% TritonX-100, 150 mM NaCl), once with high salt buffer (20 mM Tris, 2 mM EDTA, 0.1% SDS, 1% TritonX-100, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris) and 3 times with TE buffer (10 mM Tris, 1 mM EDTA). Complexes were eluted in 500 µl freshly prepared elution buffer (1% SDS, 0.1M NaHCO<sub>3</sub>) and crosslinking was reversed by addition of 5M NaCl and incubation at 65 C overnight. Samples were treated with proteinase K and phenol chloroform-extracted. DNA was ethanol precipitated in the presence of glycoblue (Ambion). Pellets were resuspended in 20 µl H<sub>2</sub>O for PCR analyses. PCR amplification of immunoprecipitated DNA was performed using primers specific for the Sp1-binding region of the p75NTR promoter as follows: forward primer 5'-TGCTGCATTGCCTTCACCC and reverse primer 5'-ACCTGCCCTCCTCATTGCAC. Control primers, amplifying a portion of the p75NTR promoter 1.5 kb distal to the Sp1-binding region were as follows: forward primer 5'-CAGTGATGTCTACCCTACCTT and reverse primer 5'-CCTCTTCAAACTACAGTCC.

## **7.3 Results**

### **7.3.1 PTEN regulation of p75NTR expression requires protein phosphatase activity**

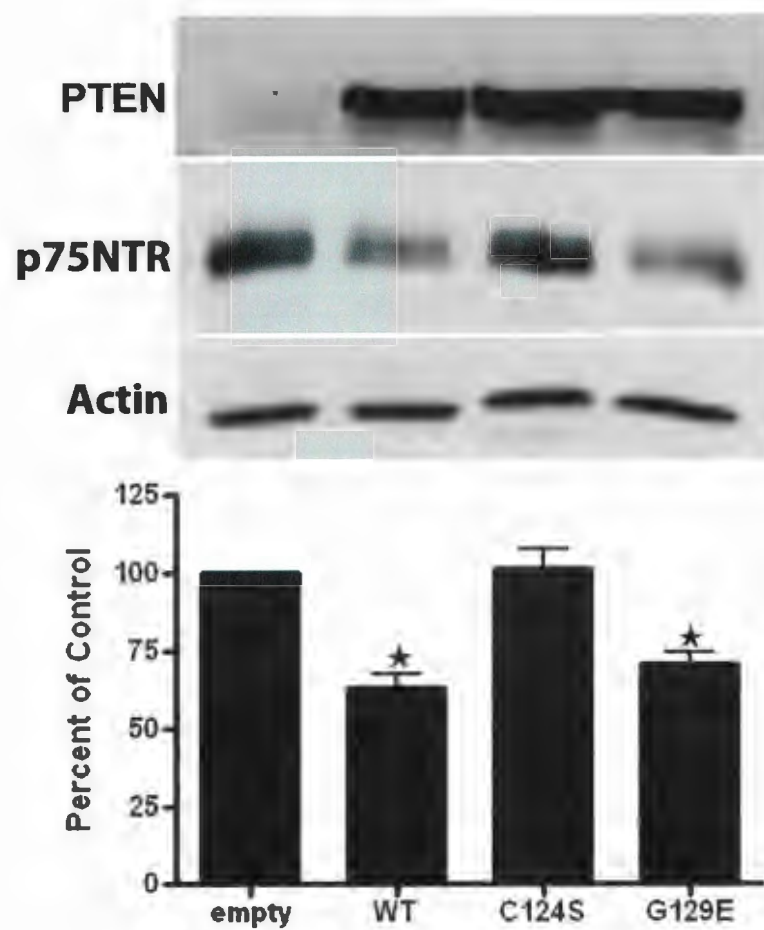
Our examination into the mechanism by which PTEN may regulate p75NTR expression was conducted using PC12 cells. Using this model, we have previously established that modulation of PTEN expression directly influenced p75NTR expression (Rankin et al, submitted). PTEN is a dual specificity phosphatase capable of dephosphorylating both lipid and protein substrates. In order to determine which of these phosphatase activities was responsible for the modulation of p75NTR expression, we overexpressed GFP-tagged PTEN constructs in PC12 cells. Overexpression of wild-type PTEN downregulated p75NTR as previously noted (Fig 7.1). The PTEN C124S mutant is devoid of phosphatase activity and failed to modulate p75NTR expression, indicating that phosphatase activity is required for PTEN regulation of p75NTR. In contrast, the G129E mutant which is lipid phosphatase dead but which retains protein phosphatase activity, significantly reduced the expression of p75NTR (Fig 7.1), suggesting that it is the protein phosphatase activity of PTEN that is able to regulate p75NTR expression.

### **7.3.2 LN-induced upregulation of PTEN decreases Sp1 binding activity**

PTEN has been found to dephosphorylate the transcription factor Sp1, thereby decreasing its DNA-binding capacity (Kang-Park et al., 2003). LN stimulation is also noted to decrease in Sp1 binding activity (Gaudreault et al., 2007). Furthermore, increased Sp1 binding activity has previously been associated with an upregulation of p75NTR (Ramos et al., 2007). To investigate the potential interaction of PTEN with Sp1,



**Figure 7.1: Protein phosphatase activity of PTEN is required for regulation of p75NTR.** PC12 cells were transfected with empty GFP, wt PTEN, or PTEN phosphorylation mutants, C124S (phosphatase dead) or G129E (lipid-phosphatase dead). p75NTR protein expression was analysed by Western blot, and found to be downregulated by wt and G129E constructs. Values expressed represent mean protein expression, relative to actin, of 3 experiments +/- SEM. \* $p < 0.05$ .



**Figure 7.1**

immunocytochemical analyses were performed initially to determine the subcellular localization of PTEN in response to a LN substrate. Cells were plated on PL or LN substrates, in the presence or absence of NGF for 24 h prior to immunocytochemical localization of PTEN. The PL substrate was associated with a cytoplasmic distribution, which appears to relocate to the membrane upon NGF stimulation (Fig 7.2A,B). Interestingly, we noted a number of cells on the LN substrate which exhibited a distinct nuclear localization of PTEN in both the presence and absence of NGF (Fig 7.2C,D) which was confirmed by Western blot analyses of fractionated cell lysates (Fig 7.2E). The LN substrate appears to relocate PTEN into the nucleus, thus indicating a potential site for its activity.

Since the LN substrate resulted in a redistribution of PTEN into the nucleus, we examined the impact of LN or PTEN overexpression on the DNA binding ability of Sp1 using electrophoretic mobility shift assays (EMSA) which were performed using nuclear extracts derived from PC12 cells cultured on PL or LN substrates for 24 h. Cold competition with an unlabelled Sp1 consensus sequence oligo, but not with a mutated Sp1 binding motif, resulted in decreased signal intensity on both PL and LN substrates (Fig 7.3) thereby confirming that the gel shift was due to the binding of Sp1 to its consensus sequence. Densitometric analysis of EMSA revealed that the LN substrate was consistently associated with decreased Sp1 binding to its radiolabelled consensus sequence (Fig 7.3A, lane 3) relative to PL (lane 2) despite the observation that the LN substrate increased the total levels of Sp1 protein detected by Western blot analysis (Fig 7.3B). To determine if the LN effect could be directly attributed to elevated levels of PTEN, PC12 cells were transiently transfected with PTEN or empty control vectors



**Figure 7.2: LN induces nuclear localization of PTEN.** Relative to PC12 cells plated on PL (A,B), cells plated on LN substrate demonstrate nuclear localization of PTEN in both presence and absence of NGF (C,D). This localization was confirmed by Western blot analysis of subcellular fractionated protein lysates (E). Scale bar -20  $\mu$ m.

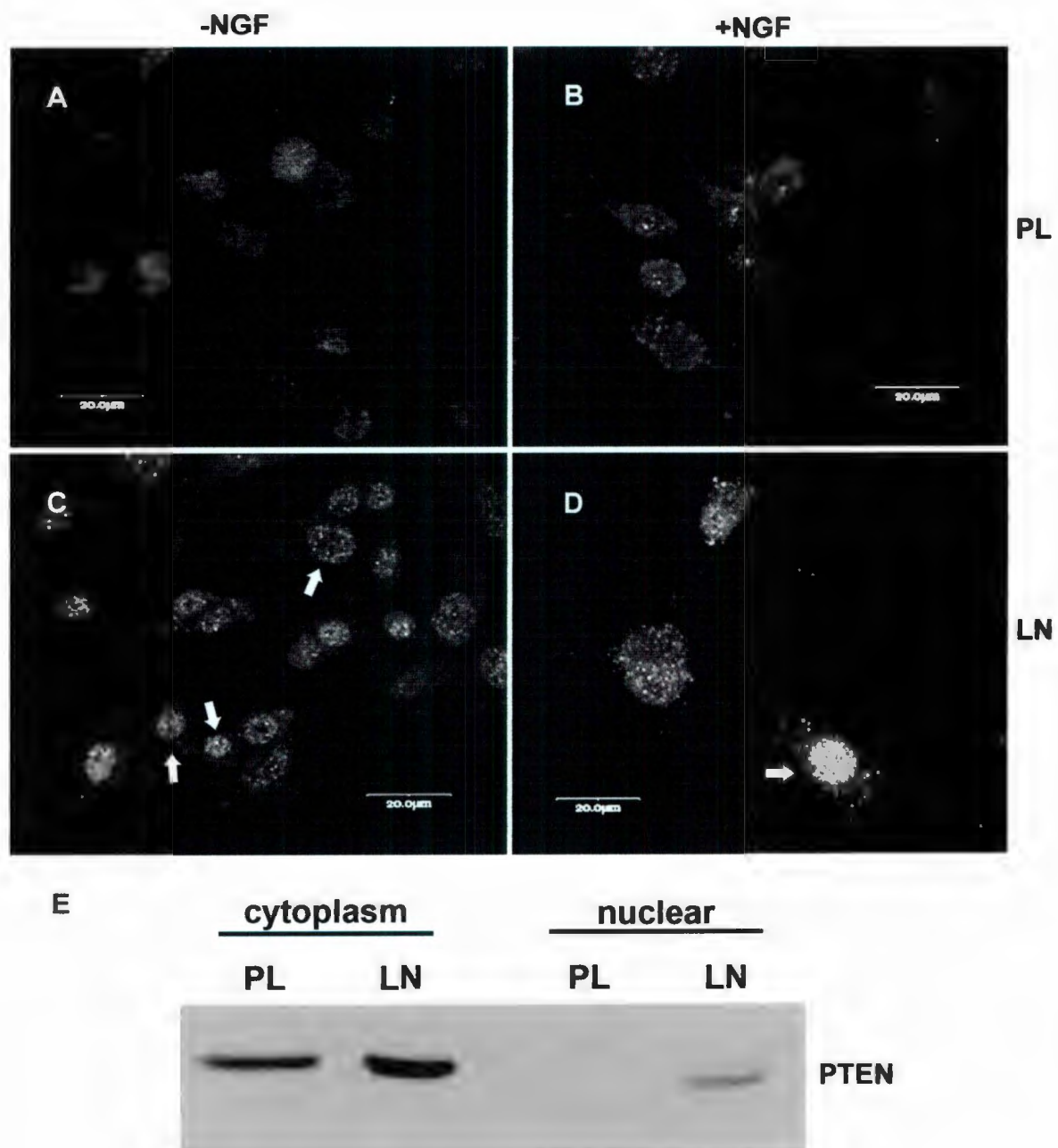
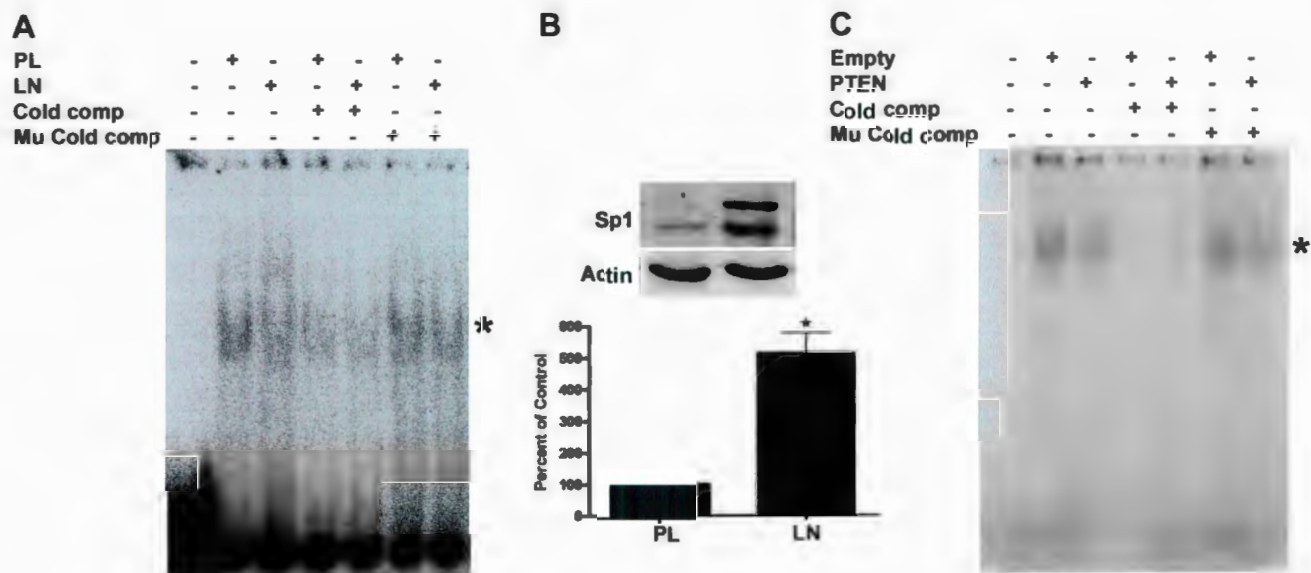


Figure 7.2

**Figure 7.3: LN-induced PTEN upregulation decreases Sp1 binding to its consensus**

**sequence.** A. PC12 cells plated on a LN substrate show decreased Sp1 binding to its consensus sequence during EMSA analysis, relative to the control PL substrate, despite an increase in Sp1 protein expression as determined by Western blotting (B).

Overexpression of PTEN also reduces Sp1 binding to its consensus sequence, relative to cells transfected with empty control vector (C). Binding can be diminished by competition with unlabeled probe, but not by competition with unlabeled mutated probe.



**Figure 7.3**



followed by EMSA analysis of Sp1 binding to its consensus sequence. PTEN overexpression resulted in a marked reduction in Sp1 binding activity (Fig 7.3C) relative to control vector.

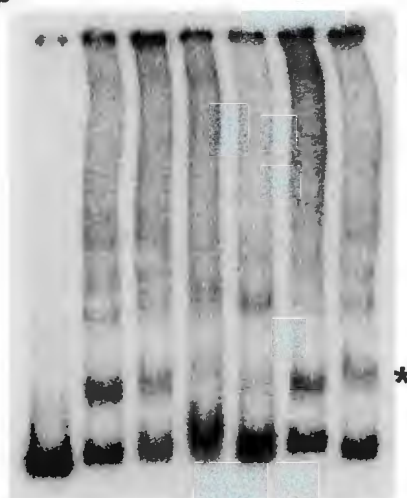
Since the capacity of Sp1 to bind to regulatory elements tends to be gene-specific, we performed an EMSA to determine the ability of Sp1 to bind specifically to the p75NTR promoter. The LN substrate resulted in significantly less binding to the radiolabelled p75NTR promoter region (Fig 7.4A, lane 3), in comparison with the control PL substrate (lane 2). To confirm that the decreased binding of Sp1 to the p75NTR promoter was due to increased levels of PTEN, we assessed Sp1 binding to the p75NTR promoter following PTEN overexpression. The binding of Sp1 to the p75NTR promoter was significantly reduced following transfection with a PTEN construct relative to the empty vector control (Fig 7.4B). Therefore, both the LN substrate and the overexpression of PTEN can decrease the ability of Sp1 to bind to its consensus sequence on the p75NTR promoter.

To determine if LN results in decreased binding of Sp1 to the p75NTR promoter in an *in vivo* situation, we utilized chromatin immunoprecipitation (ChIP) to directly assess the physical interaction of Sp1 with the p75NTR promoter in response to a LN substrate, or PTEN overexpression. PC12 cells plated on a LN substrate for 24 h exhibited a significant reduction in the amount of Sp1-precipitated p75NTR promoter (Fig 7.5A, lane 4) in comparison to the control PL substrate (Fig 7.5A, lane 3). Similarly, PTEN overexpression resulted in significantly reduced detection of Sp1-precipitated p75NTR DNA (Fig 7.5B, lane 4) in comparison with empty vector control (Fig 7.5B, lane

**Figure 7.4: LN-induced PTEN upregulation decreases Sp1 binding to the p75NTR promoter.** A. PC12 cells plated on a LN substrate show decreased Sp1 binding to the p75NTR promoter during EMSA analyses relative to the control PL substrate. This binding can be diminished by competition with unlabeled probe, but not by competition with an unlabeled mutated probe. B. Overexpression of PTEN also reduces Sp1 binding to the p75NTR promoter, in comparison to transfection with empty control vector.

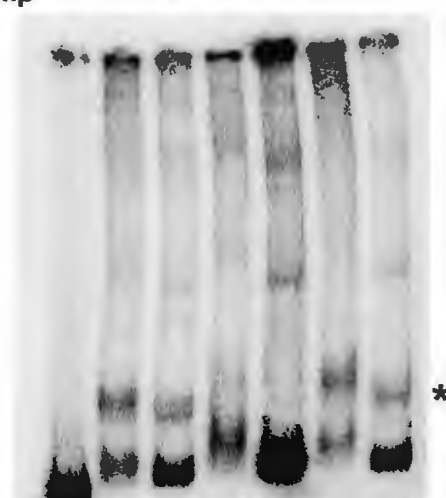
**A**

PL	-	+	-	+	-	+	-
LN	-	-	+	-	+	-	+
Cold comp	-	-	-	+	+	-	-
Mu Cold comp	-	-	-	-	-	+	+



**B**

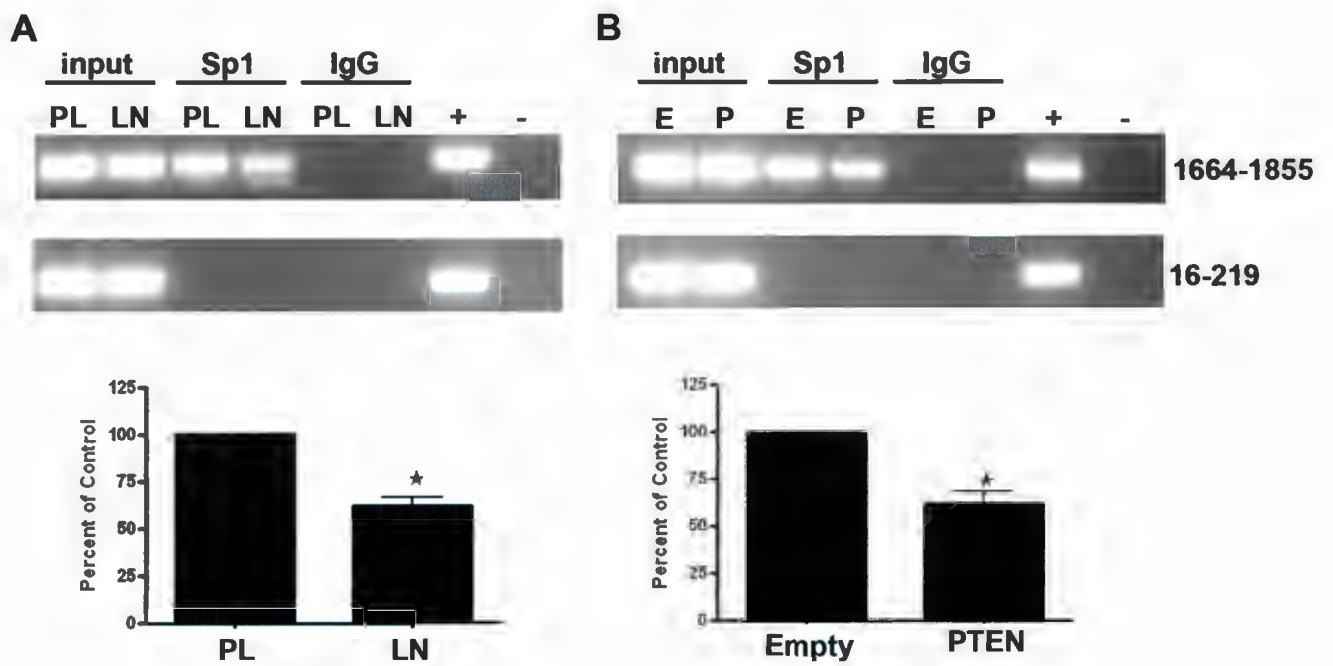
Empty	-	+	-	+	-	+	-
PTEN	-	-	+	-	+	-	+
Cold comp	-	-	-	+	+	-	-
Mu Cold comp	-	-	-	-	-	+	+



**Figure 7.4**

**Figure 7.5: LN-induced PTEN upregulation decreases Sp1 binding to the p75NTR promoter *in vivo*.** A. LN exposed PC12 cells subjected to Sp1 ChIP show decreased PCR amplification of the Sp1 binding region of the p75NTR promoter (1664-1855), but not a distal fragment (16-219) relative to the control PL substrate. B. Overexpression of PTEN (P) also reduces amplification of the Sp1 binding region of the p75NTR promoter (1664-1855), in comparison to transfection with empty vector (E). \* $p < 0.01$ .





**Figure 7.5**

3) thus confirming a link between the level of PTEN expression and the capacity of Sp1 to bind to the p75NTR promoter.

Finally, we ascertained whether this mechanism of p75NTR regulation was valid in primary neurons which also display an inverse PTEN-p75NTR relationship. We have previously determined that cerebellar granule neurons (CGN) also upregulate PTEN expression in response to LN stimulation, leading to a downregulation of p75NTR (Rankin et al. In preparation). Thus, to assess the effects of LN on Sp1 binding activity, CGN were purified from post-natal day 8 rats and were plated on PL or LN substrates. EMSA analysis shows that LN decreases Sp1 binding to its consensus sequence (Fig 7.6A, lane 3) and to the p75NTR promoter (Fig 7.6B, lane 3) in CGN. Furthermore, ChIP assays confirmed a decreased interaction between Sp1 and the p75NTR promoter in CGN plated on LN compared to the control PL substrate (Fig 7.6C). These results suggest that the regulatory mechanism responsible for PTEN-mediated regulation of p75NTR observed in neuronal cell lines may also be operative in primary neurons.

#### **7.4 Discussion**

This study presents a mechanism by which a phosphatase, PTEN, can control the transcriptional regulation of a pleiotropic receptor p75NTR as directed by the ECM in an integrin-dependent manner. We have previously shown that PTEN expression inversely correlates with p75NTR expression (Rankin et al, submitted). Furthermore, PTEN overexpression is associated with a decrease of p75NTR, and PTEN siRNA can increase p75NTR. Here we investigated the potential mechanisms for this and report that LN

**Figure 7.6: LN reduces the binding activity of Sp1 in CGN.** CGN plated on a LN substrate show decreased Sp1 binding to its consensus sequence (A) and the p75NTR promoter (B) relative to the control PL substrate during EMSA analyses. ChIP analyses confirm decreased amplification of the Sp1 binding domain of the p75NTR promoter (1664-1855), but not a distal fragment (16-219) (C) induced by the LN substrate.

\* $p < 0.01$ .

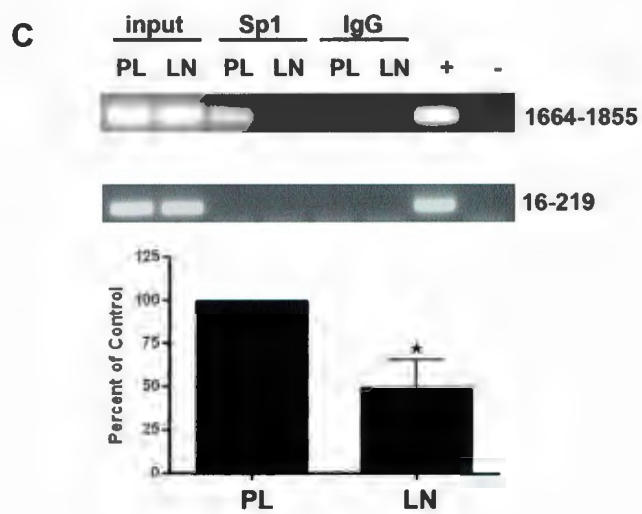
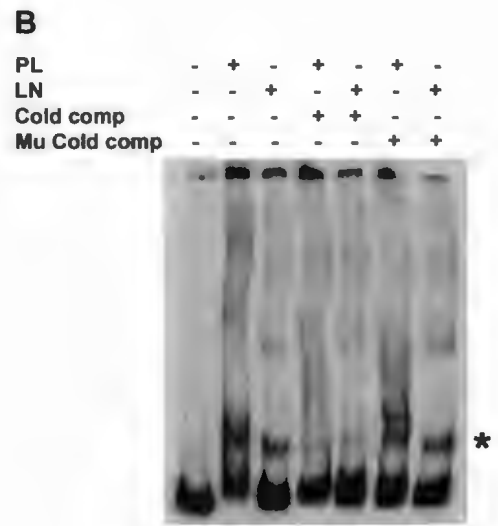
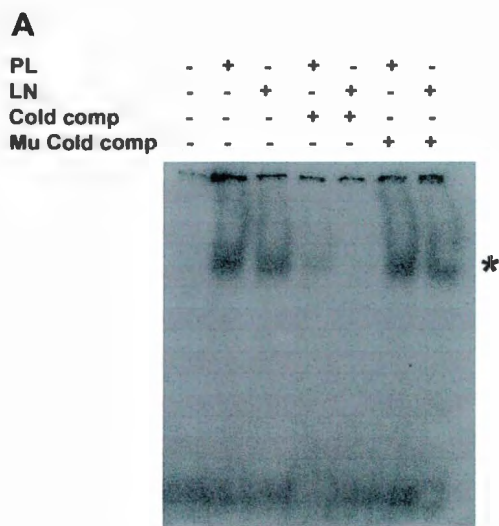


Figure 7.6



induces PTEN to relocate to the nucleus, where it could dephosphorylate Sp1, leading to a decreased ability to bind to the p75NTR promoter resulting in reduced p75NTR expression.

PTEN is capable of dephosphorylating both lipid and protein substrates. The lipid phosphatase activities of PTEN have been intensively studied. PTEN is a well known antagonist of PI3 kinase, with its primary substrate believed to be PIP<sub>3</sub>, though notably, it also has a minor effect on PLC $\gamma$  (Alvarez-Breckenridge et al., 2007), a phospholipase which increases p75NTR expression upon ligand binding to the Trk receptor (Rankin et al., 2008). The protein phosphatase activities of PTEN are less understood. Proposed targets include Fak and Shc (Tamura et al., 1998), however these may be cell type and context-specific. Using a mutated PTEN construct (G129E) that is lipid phosphatase dead but retains its activity against protein substrates, we demonstrate that the protein phosphatase activities are necessary to decrease p75NTR expression while mutants with a total loss of phosphatase activity could not. Our immunocytochemical studies revealed nuclear localization for PTEN induced by the LN substrate. Nuclear localization of PTEN is a well-documented phenomenon (Baker, 2007) which suggests regulatory functions but an exact role is largely unknown. PTEN is also noted to dephosphorylate transcription factor Sp1 (Kang-Park et al., 2003), the phosphorylation status of which directly impacts its ability to bind to some DNA promoter regions (Bouwman and Philipsen, 2002; Li et al., 2004). Previous reports confirm that LN decreases Sp1 activity (Gaudreault et al., 2007; Gehler et al., 2004; Gentry et al., 2004; Glowacka et al., 1992; Goettl et al., 2004), and Sp1 is highlighted to play a role in the induction of p75NTR expression in response to changes in osmolarity (Ramos et al., 2007). It is likely that the

LN-induced decrease of Sp1 binding is mediated by PTEN protein phosphatase activities resulting in a decrease of p75NTR expression. Finally, we confirm that this relationship occurs in CGN where LN-mediated changes in PTEN and p75NTR have been shown to influence the developmental migration of these neurons to their final anatomical and functional location.

#### **7.4.1 Speculation**

PTEN is often lost or mutated in human cancer. The associated loss of function may upset the balance of physiological processes including cell cycle, growth, differentiation and motility. As such, decreased PTEN expression is associated with enhanced invasive potential of metastatic glioma (Knobbe et al., 2002). Notably, PTEN normally prevents this invasion through its protein phosphatase actions (Downes et al., 2007). An increase in p75NTR has independently been noted to enhance the invasion of metastatic glioma (Johnston et al., 2007). We hypothesize that the mechanism presented here may contribute to the pathogenesis of glioma. Furthermore, since Sp1 is a highly prevalent transcription factor controlling the expression of a multitude of cellular proteins, the current study may be of relevance for many research paradigms.

## **Chapter 8: TrkA modulates the expression of PTEN resulting in alterations in motility and growth potential**

### **8.0 Summary**

The regulation of p75NTR expression is important to multiple cellular processes including survival, development and maintenance of the nervous system. Constitutive expression of p75NTR has been previously shown to be influenced by the high affinity NGF receptor TrkA. More recently, a role for the extracellular matrix in regulation of p75NTR has been ascribed to a laminin-mediated upregulation of the phosphatase, PTEN. I now report that the individual phosphorylation sites of TrkA modulate both constitutive PTEN expression and the capacity of the cells to respond to laminin by downregulation of p75NTR and subsequent depression of Rho activity. Finally, I demonstrate that TrkA mutations associated with decreased levels of PTEN functionally impair the cell's ability to remodel the actin cytoskeleton thus negatively impact both growth and motility.

### **8.1 Introduction**

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a well-known tumor suppressor expressed by a wide variety of cells; its functions are frequently non-redundant requiring that PTEN expression be under tight regulatory control (Salmena et al., 2008). PTEN is a dual specificity phosphatase, capable of dephosphorylating both lipid and protein substrates. The lipid phosphatase actions of PTEN are well characterized for antagonism of the PI3K-Akt survival pathway, in which PTEN



dephosphorylates PIP<sub>3</sub>, thus preventing downstream phosphorylation of Akt and its associated cascades, often inducing apoptosis (Downes et al., 2001; Knobbe et al., 2002; Salmena et al., 2008). The protein phosphatase substrates of PTEN are still largely unknown, though recently we have demonstrated that a protein phosphatase action of PTEN is necessary for the downregulation of p75NTR (Rankin et al. 2008b).

p75NTR is the low affinity pan-neurotrophin receptor well known for its essential roles in the development and maintenance of the nervous system (Blochl and Blochl, 2007). In the presence of neurotrophins, p75NTR signals co-ordinately with the high affinity receptor, a specific member of the Trk family of receptor tyrosine kinases, to promote neurite outgrowth by enhancing the phosphorylation of Trk itself and its downstream signalling intermediates (Roux and Barker, 2002).

The growth-promoting behaviour of p75NTR is, however, cell type and context specific, and depends upon the ratio of Trk to p75NTR (Althaus and Kloppner, 2006; Gatzinsky et al., 2001; Yan et al., 2002) and the presence of neurotrophins. In the absence of neurotrophins, p75NTR can inhibit growth through a variety of mechanisms including constitutive activation of Rho (Yamashita et al., 1999), a small GTPase that regulates the dynamics of the actin cytoskeleton, and by transducing myelin-derived inhibitory signals (Wang et al., 2002a). This growth inhibiting behaviour is dependent upon co-receptor availability and p75NTR expression levels.

Ligation of the nerve growth factor (NGF) receptor, TrkA, initiates receptor homodimerization which results in the transphosphorylation of specific tyrosine residues on the cytoplasmic tail, subsequently initiating signalling cascades for growth and survival (Kaplan and Miller, 2000; Reichardt, 2006). Phosphorylation of Trk Y490



creates a docking site for Shc, an adapter protein which initiates the Ras-MEK-MAPK cascade (Obermeier et al., 1993b). Phosphorylation of Trk Y785 creates a site of interaction for PLC $\gamma$ , which activates downstream PKC and MAPK signalling (Obermeier et al., 1993a). Additionally, phosphorylation of the Trk cytoplasmic domain is associated with the activation of PI3K signalling for survival. We have previously shown that TrkA, regulates the constitutive expression of p75NTR (Rankin et al., 2005), and that Trk ligation results in the upregulation of p75NTR via a PLC $\gamma$ -PKC $\delta$ -dependent mechanism to promote growth (Rankin et al., 2008). More recently, we have elucidated a second mechanism by which the expression level of p75NTR can be manipulated. This cascade is initiated by extracellular matrix molecule laminin (LN), which upregulates PTEN (Rankin et al. 2008b). PTEN then translocates to the nucleus where it has been shown by others to dephosphorylate transcription factor Sp1 (Kang-Park et al., 2003). PTEN overexpression has been demonstrated to decrease the binding ability of Sp1 to the p75NTR promoter thus decreasing p75NTR expression to again promote growth and motility (Rankin et al. Manuscript submitted).

The goal of this study was to determine if there exists any interaction between these two p75NTR regulatory mechanisms. As we have previously demonstrated that a LN substrate and the associated high PTEN levels had no effect on TrkA expression (Rankin et al. 2008b), we specifically sought to determine if TrkA signalling plays a role in the regulation of PTEN expression. Our results show that TrkA activation decreases PTEN expression, and that PTEN manipulation by TrkA mutations can regulate the growth and motility potential of PC12 cells in response to a LN substrate. p75NTR is a pleiotropic receptor likely to be tightly regulated in terms of spatial, temporal and

molecular distribution. This link adds another layer of complexity to the regulation of p75NTR and its contribution to the growth response.

## **8.2 Materials and Methods**

### **8.2.1 Cell culture and differentiation:**

The experimental model consisted of wild-type rat pheochromocytoma (PC12) cells, and 4 mutated PC12 derivative cell lines (gifts from Dr. David Kaplan, Hospital for Sick Children, Toronto, ON). PC12nnr5 cells were derived by mutating parental PC12 cells using ethyl methanesulfonate (EMS), as described by Green et al. (1986). PC12 derivatives expressing mutated TrkA receptors (Y490F, Y785F, and Y490/785F) were created by Stephens et al. (1994). Briefly, PC12nnr5 cells, which lack endogenous TrkA, were transfected to express a TrkA receptor that had been altered via site-directed mutagenesis to abrogate either one or both of the autophosphorylation sites of the cytoplasmic tail.

Cell lines were maintained on rat-tail collagen-coated tissue-culture flasks in RPMI 1640 medium (Invitrogen, Burlington ON) supplemented with 10% horse serum (Invitrogen), 5% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin/glutamine solution (Invitrogen). In the case of mutated lines Y490F, Y785F and Y490/785F, media was additionally supplemented with Geneticin G418 (4 µl/ml) to ensure continued selection of the mutant population. Cells were incubated at 37°C in 5% CO<sub>2</sub>, and cultured to 80% confluence prior to trypsinization for subculturing purposes. Cells were



differentiated by exposure to NGF (100 ng/ml) for 2 days in low serum conditions to prime, followed by exposure to NGF (100 ng/ml) for 5 days in the absence of serum.

### **8.2.2 Plasmids and Transfection:**

Full length rat TrkA coding sequence was amplified from cDNA using PCR forward primer 5'-CTCGAGATGCTGCGAGGCCAGC and reverse primer 5'-GATATCGTGCCCAGAACGTCC incorporating XhoI and EcoRV restriction enzyme sequences, respectively. PCR amplicons were cloned into the TOPO TA cloning vector PCR2.1 (Invitrogen), and nucleotide sequence determined by automated sequencing prior to directional insertion into the eukaryotic expression vector pcDNA3.1 (Invitrogen) engineered to include a carboxy terminal orange fluorescent protein tag.

Empty pcDNA3.1-orange control or pcDNA3.1-TrkA-orange vectors were transfected into PC12 cells and derivatives using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions.

### **8.2.3 TrkA anti-sense oligos:**

TrkA sense and antisense oligos were synthesized by the central facility of Institute for Molecular Biology and Biotechnology, McMaster University as per the following sequences: Control 1: 5'-ATGGCGCGGCTCTGGGGCTTA, Control 2: 5'-GAACGGATTACCCGTCAGGAT, Antisense 1: 5'-CCGCTGGCCTCGCAGCATCGC, Antisense 2: 5'-CACATCATTCTCTGCCCAGCA. Oligos were stored in lyophilized format at -20 C until use, when they were reconstituted for use at a final concentration of 2.5  $\mu$ M each. Oligos were delivered to the cells using lipofectamine 2000 as per the manufacturer's instructions.

#### **8.2.4 Western blot analysis:**

For Western analyses, cells were harvested in the presence of sodium orthovanadate (100 mM in TBS) and subsequently subjected to lysis (10% glycerol, 1% NP-40, sodium vanadate, sodium fluoride, sodium dodecyl sulphate (SDS) and 1 protease inhibitor cocktail tablet (Roche Scientific, Laval, QC) overnight at 4° C prior to centrifugation (10,000 g, 5 min). A BCA protein assay (Pierce, Rockford IL) was used to determine protein concentration and equivalent amounts of protein (50 µg) were electrophoresed on 8% SDS-polyacrylamide gels. Protein was subsequently transferred to nitrocellulose membranes that were then exposed to Ponceau red to ensure equal protein loading. After washing in TBS, blots were blocked in 3% non-fat dry milk for 1 h at room temperature, and then incubated with a primary antibody directed toward either p75NTR (Upstate), PTEN (Cell Signalling), TrkA (Upstate) or actin (Sigma) overnight at 4° C. A final incubation with HRP-conjugated secondary antibody (AP307P, AP308P; Chemicon, Temecula, CA) for 1 h at room temperature was followed by visualization using an ECL method. Each experiment was repeated three times, subjected to densitometry and normalized to actin to ensure equal protein for comparison. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego CA) with significance being determined using one-way ANOVA testing.

#### **8.2.5 Real-time RT-PCR:**

For real time RT-PCR analysis, cells were subcultured on Poly-D-Lysine (PL)- or Laminin (LN)-coated 12-well plates under serum-starved conditions for 24 hours. RNA



was isolated using Trizol reagent (Invitrogen) as per the manufacturer's instructions and was subsequently treated with DNase (Ambion; Austin TX) to remove traces of contaminating DNA. RNA (2 µg) was reverse transcribed to cDNA using MMLV reverse transcriptase (200 U for 30 minutes at 37 C; Invitrogen) prior to use as template for real-time PCR amplification using the following PCR primer pairs: p75NTR forward 5'-TGCATCTGAGCTGGTGTCTGTCTT, p75NTR reverse 5'-TGCGTACAATGCTCCTGGTCTCTT, PTEN forward 5'-ATTCGACTTAGACTTGACCT, PTEN reverse 5'-ACCAGTCCGTCCTTTC, 28S forward 5'-GACCAAGGAGTCTAACGC, 28S reverse 5'-GTACGCTCGTGCTCCA. PCR amplification was performed using the Roche LightCycler (Roche) and quantified using SYBR green I. p75NTR and PTEN mRNA expression levels were subsequently normalized using the housekeeping gene, 28S.

#### **8.2.6 Rho activity assay:**

The activation of RhoA was assessed using a pull-down assay kit (Upstate, Charlottesville, VA), in accordance with the manufacturer's instructions. Briefly, cells were harvested and lysed. Rhotekin-bound beads were added to cleared lysate and rotated for 1 hour at 4°C prior to pelleting, washing, and boiling in Laemmli buffer with DTT. Rhotekin-bound Rho proteins were detected by Western blot analysis using a polyclonal anti-Rho antibody (Upstate). The total amount of RhoA in cell lysate was used as a control for the cross-comparison of Rho activity.

### **8.2.7 Immunocytochemistry:**

For immunocytochemical studies, cells were trypsinized and subcultured serum-free on PL or LN-coated 16-well chamber slides for 24 hours prior to fixation in 4% paraformaldehyde for 15 minutes. Cells were then permeabilized with 0.1% Triton-X-100 and blocked with 10% normal goat serum in PBS for 1 hour at room temperature. Cells were incubated with either a monoclonal antibody directed against total tubulin (Sigma) or the combination of a mouse monoclonal antibody directed against p75NTR (MC192, Oncogene) and a rabbit monoclonal antibody directed against PTEN (Cell Signaling) for 16 hours at 4° C. This was followed by incubation with Cy2- and Cy5-tagged secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) for 1 hour at room temperature. Finally, slides were coverslipped with glycerol, and the receptor expression patterns were visualized using confocal laser scanning microscopy.

### **8.2.8 Growth quantification:**

All measurements of growth were obtained using images of PC12 cells immunostained for total tubulin (Sigma). Individual cell tracings were performed using NIH Image J software. Only cells for which we were able to unambiguously identify the associated neurites were chosen for tracing analysis. Neurite length is a measure of the longest neurite extending from each cell (n>250 cells per condition). Data were imported into a graphing and statistical analysis program (Prism 4, Graphpad Corp.) for further analysis.

### **8.2.9 Motility Assay:**

Motility was assessed using a previously described assay (Rankin et al., 2006). In brief, cells were plated in a monolayer on a PL-coated glass coverslip. Once cells became attached, coverslips were inverted onto 12-well tissue culture plates coated with PL or LN substrates. Cells were allowed to migrate from underneath the inverted coverslip into the migratory field for 36h prior to image acquisition. Cell migration was quantified using a semi-automated cell counter plugin for Image J (NIH).

## **8.3 Results**

### **8.3.1 Manipulation of TrkA modulates PTEN expression**

TrkA has been previously shown to regulate the constitutive expression of p75NTR (Rankin et al., 2005). Additionally, p75NTR expression can be regulated by the phosphatase, PTEN (Rankin et al. 2008b). Since TrkA and PTEN have been independently shown to regulate p75NTR, I assessed whether these regulatory pathways may intersect, specifically if TrkA plays a role in the modulation of PTEN expression. For this purpose, PC12nnr5 cells, which lack endogenous TrkA expression, were transiently transfected with a full-length TrkA-orange fluorescent construct or empty vector control. It has been previously demonstrated that expression of TrkA with a C-terminus GFP tag in PC12nnr5 cells does not interfere with NGF-induced TrkA phosphorylation or the initiation of signalling cascades (Jullien et al., 2003). Western blot analyses of protein lysates collected 48 h post-transfection revealed that the overexpression of TrkA significantly reduced PTEN expression levels relative to the



control empty vector (Figure 8.1A). Since PC12 nmr5 cells lack endogenous TrkA and subsequently express constitutively higher levels of PTEN relative to the parental PC12 cells, I determined whether abrogation of endogenous TrkA expression is sufficient to increase PTEN expression. Thus, PC12 cells were transfected with antisense oligos directed against TrkA, and cell lysates were analyzed by Western blot. Transfection with TrkA antisense, but not control sense oligos, resulted in a significant increase in PTEN expression (Figure 8.1B) in parental PC12 cells. This finding was replicated using a pharmacological inhibitor of TrkA, K252a (200 nM), which induced the upregulation of PTEN within an hour of inhibition (Figure 8.1C). Since we have previously demonstrated that PTEN expression downregulates p75NTR (Rankin et al. 2008b), and that K252a downregulates p75NTR (Rankin et al. 2008), we examined the temporal relationship of K252a induction of PTEN with changes to p75NTR. The downregulation of p75NTR was subsequently detectable 6 hours after the initiation of TrkA inhibition (Figure 8.1C). I have further determined that p75NTR overexpression does not impact expression of PTEN (data not shown). Taken together, these results suggest a regulatory role for TrkA in the constitutive expression of PTEN, which occurs upstream of changes to p75NTR expression.

### **8.3.2 PTEN expression is influenced by mutation of TrkA phosphorylation sites**

TrkA possesses two phosphorylatable tyrosines outside of the kinase domain which, upon phosphorylation, initiate well described signal transduction cascades (Kaplan and Miller, 2000; Lei and Parada, 2007). Since TrkA constitutively regulates PTEN expression in the parental PC12 cells, it remained possible that PTEN regulation could

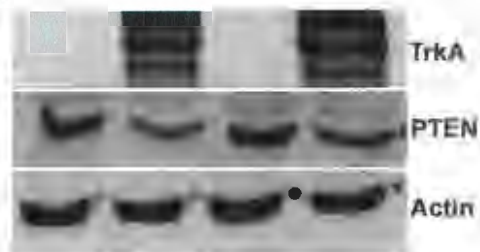


**Figure 8.1: PTEN expression is modulated following manipulation of TrkA.**

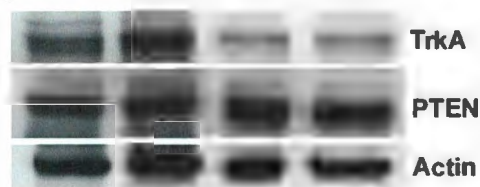
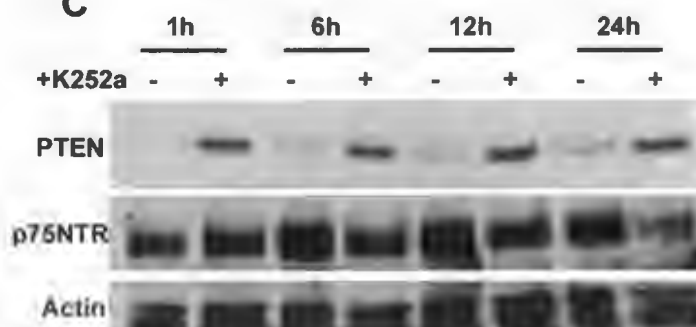
PC12nr5 cells which lack endogenous TrkA (A) were transiently transfected with empty vector or fluorescently tagged full length TrkA prior to Western blot analyses which showed decrease PTEN expression in response to TrkA overexpression. Conversely, transfection of PC12 cells with TrkA antisense oligos (B) increased PTEN expression relative to transfection with Trk sense oligos. Values expressed represent the mean expression level, relative to actin,  $\pm$  SEM as determined in three experiments, where  $*p < 0.05$ ,  $**p < 0.01$  as determined by one way ANOVA, and expressed as a percentage of the PL control condition.. Culturing PC12 cells in the presence of Trk inhibitor, K252a, resulted in the early upregulation of PTEN, detectable within an hour of exposure, and the subsequent downregulation of p75NTR, detectable 6 h following exposure (C).

**A**

PL	+	+	-	-
PL+NGF	-	-	+	+
Empty vector	+	-	+	-
TrkA-orange	-	+	-	+

**B**

NGF	-	+	-	+
Sense	+	+	-	-
Trk antisense	-	-	+	+

**C****Figure 8.1**

occur through either of these TrkA-associated signalling cascades. Therefore, I assessed constitutive expression of PTEN in a series of PC12 mutant cell lines that stably express TrkA which has been mutated by site-directed mutagenesis to abrogate either one, or both of these phosphorylatable tyrosine residues. Western blot analyses of the parental and PC12 derivatives reveals that in the absence of TrkA (PC12nnr5 cells), there is an increase in PTEN relative to the parental PC12 cell line (Figure 8.2A). While there is no detectable PTEN expression in cells expressing a mutant TrkA with the inactive Y490 phosphorylation site, analysis of cells carrying a mutated Y785 site showed similar PTEN expression levels as observed in the TrkA deficient PC12nnr5 cells (Figure 8.2A). Mutation of both tyrosine phosphorylation sites restores PTEN expression to levels comparable to those observed in the parental PC12 cell line (Figure 8.2A). This pattern of expression was confirmed by real-time RT-PCR analyses (Figure 8.2B). These results further indicated that while the Y490 TrkA mutant cell lines did not express PTEN protein detectable by Western blot analysis, mRNA expression was minimally detected, although at levels approximately 10-fold less than those observed in the wild type PC12 cells. In support of our recent report which suggested a regulatory role for PTEN in the expression of p75NTR (Rankin et al. 2008b), the current data are in line with our initial report which demonstrated that Y490 mutant PC12 cells constitutively express significantly greater p75NTR in comparison with the parental PC12 line (Rankin et al., 2005). Furthermore, the efficacy of K252a in the induction of PTEN (Figure 8.1C) suggests that there is constitutive activation of TrkA in the absence of ligand, a phenomenon previously reported to be associated with high levels of Trk expression or overexpression (Leoni and Valtorta, 2002). To determine if the overexpression of the

**Figure 8.2: Mutation of individual phosphorylation sites of TrkA modulates PTEN expression.** Parental PC12 cells, PC12nnr5 cells, or PC12 derivative cell lines expressing TrkA Y490F, Y785F or both Y490F/Y785F mutations, were analyzed by Western blotting (A) or real-time RT-PCR (B) for PTEN expression levels. Relative to the parental line, PC12nnr5 and Y785F cells exhibit increased PTEN, while Y490F cells lack detectable PTEN protein expression and display significantly reduced PTEN mRNA transcription. Y490F/Y785F cells did not significantly differ from the parental PC12 line. To determine if the changes in PTEN expression were due to constitutive TrkA activation, cell lysates were analyzed for phosphorylation of TrkA at Y674 (C), and show low level activation relative to a positive control (5 min NGF stimulation) in each cell line except PC12nnr5. Arrow indicates TrkA band. Data represent mean values  $\pm$  SEM obtained from three independent experiments following protein normalization to actin, or mRNA expression normalized to 28s, and are expressed relative to the control PC12 parental line where  $*p < 0.01$  as determined by one way ANOVA.



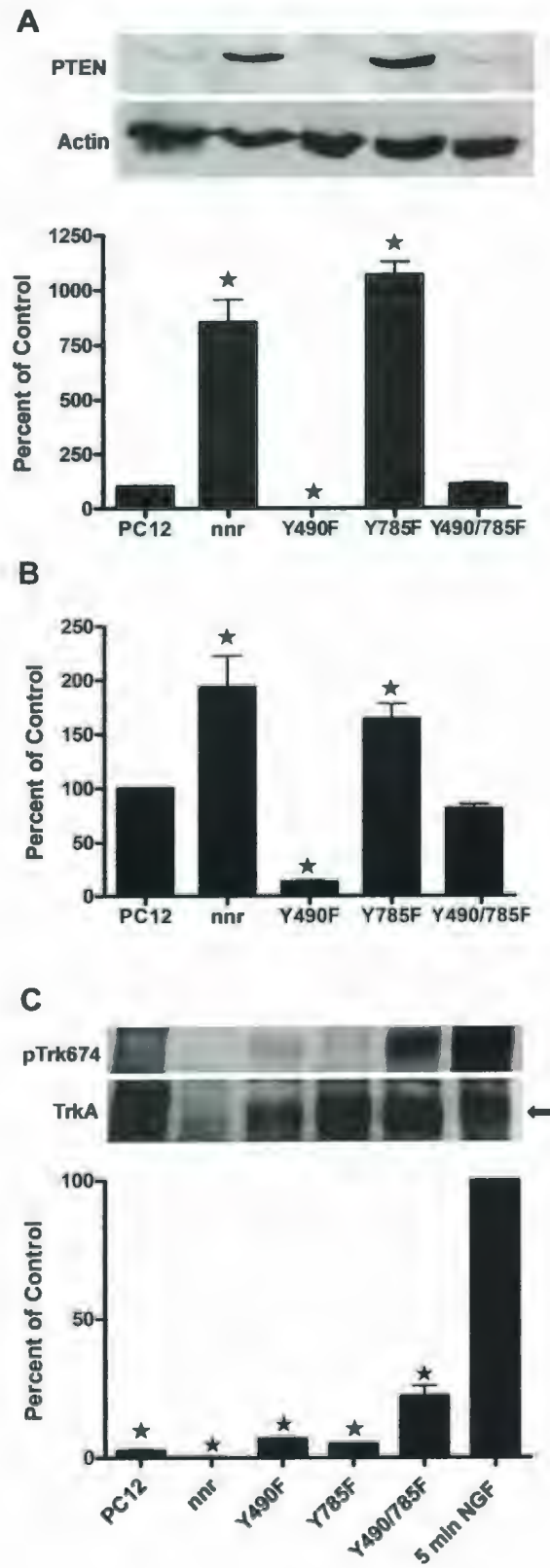


Figure 8.2

mutated Trk receptors results in their constitutive activation, I subjected lysates to immunoblotting for TrkA phosphorylation of Y674 of the activation domain. Results indicate that there is in fact low level activation of TrkA detectable in all cell lines except PC12nnr5 (Figure 8.2 C). Thus, an inverse correlation of cellular expression levels of PTEN and p75NTR are consistently observed and may be ascribed to alterations in the TrkA signal transduction cascade.

### **8.3.3 TrkA mutations modulate the capacity of LN to regulate PTEN, p75NTR and the activity of Rho**

We have recently demonstrated that LN-mediated downregulation of p75NTR results in enhanced neurite outgrowth, and that LN-induced PTEN expression is a key component of this downregulation (Rankin et al. 2008b). To determine whether this LN-induced cascade is impacted by TrkA-mediated alterations in PTEN expression as we have noted above, PC12 cells and TrkA mutated derivatives were plated on PL or LN substrates for 24 h and subsequently analyzed by Western blotting. Wild type and PC12 derivatives demonstrated increased PTEN in response to LN, with the exception of the Y490F TrkA mutant cell line, in which LN failed to induce expression of PTEN (Figure 8.3A). In agreement with our previously established signalling cascade (Rankin et al. 2008b), LN significantly reduced p75NTR expression in those cell lines which had the ability to upregulate PTEN (Figure 8.3B). In contrast, Y490F TrkA mutants which failed to upregulate PTEN in response to the LN substrate, did not demonstrate reduced p75NTR expression following exposure to LN (Figure 8.3B). These results were confirmed by real-time RT-PCR analyses (Figure 8.3C-D) and by immunocytochemistry

**Figure 8.3: TrkA modulates the capacity of PTEN to upregulate in response to LN and subsequently regulate p75NTR.** Parental PC12 cells, PC12nnr5 cells, or PC12 derivative cell lines expressing either TrkA Y490F, Y785F or both Y490F/Y785F mutations were subcultured on PL or LN for 24 h prior to Western blot (A, B) or real-time RT-PCR analyses (C, D) of PTEN (A, C) or p75NTR (B, D) expression. LN induces an upregulation of PTEN and converse downregulation of p75NTR in all cell lines excluding those with the TrkA Y490F mutation. This relationship was confirmed by immunocytochemical analyses of the 5 cell lines plated on PL or LN substrates, and representative images are depicted (E – T). Densitometric analyses of protein expression were normalized to actin, while real-time RT-PCR analyses were normalized using 28s. Values expressed in A – D represent the mean expression levels obtained from 3 independent experiments +/- SEM where \* $p < 0.01$  as determined by one way ANOVA.

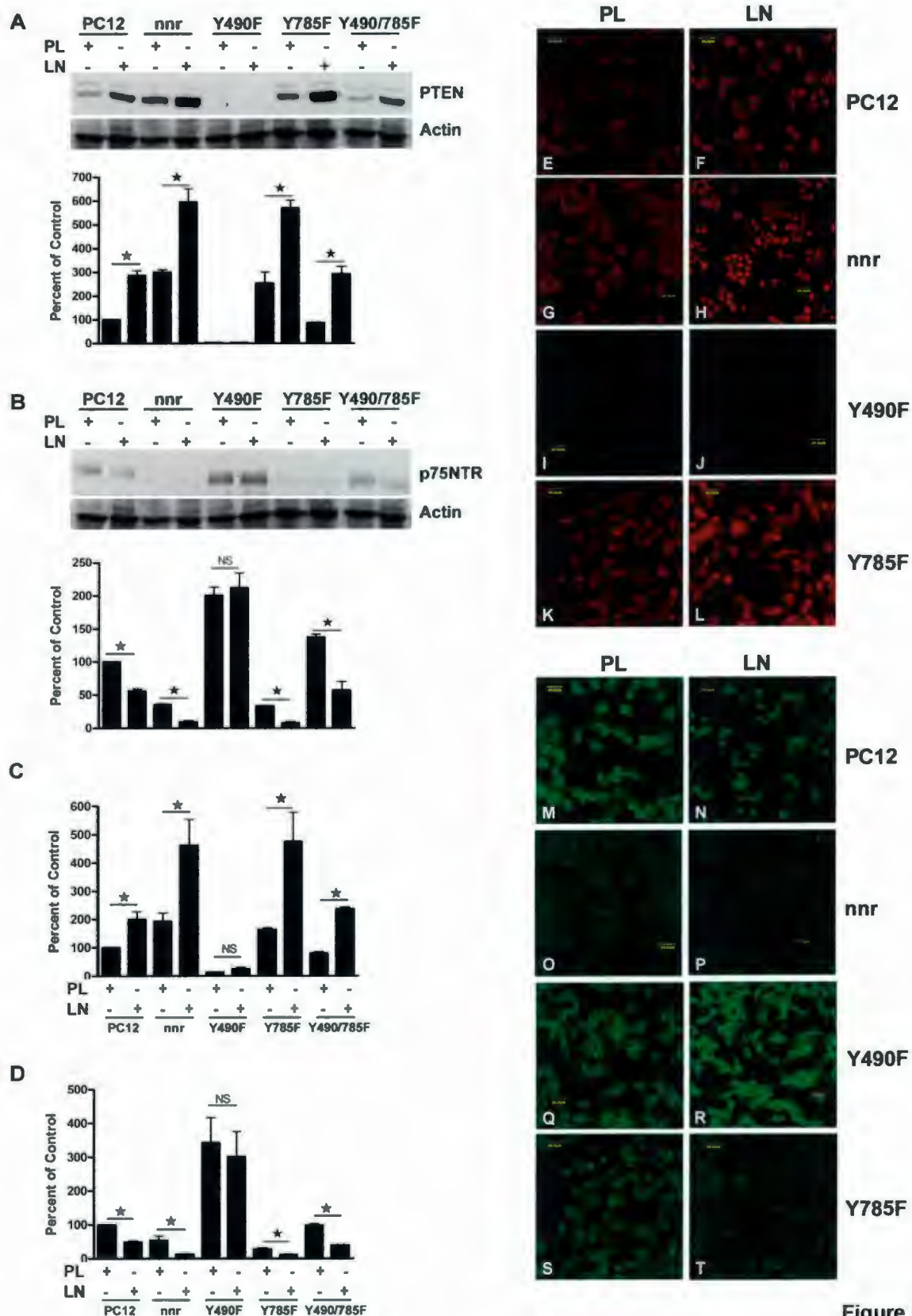


Figure 8.3



for PTEN and p75NTR localizations in response to a LN substrate, relative to PL (Figures 8.3E-3T). Cells expressing TrkA with the double mutation (Y490/785F) do not significantly differ from wild-type in PTEN or p75NTR expression or localization, and thus are not shown in Figure 8.3E-T photomicrographs. Together these results demonstrate that TrkA plays a role in the response of PC12 cells to a LN substrate wherein PTEN modulation may represent a key signalling intermediate.

#### **8.3.4 Biological response to LN is altered in TrkA mutant PC12 cells**

Since p75NTR constitutively activates Rho (Yamashita et al., 1999), and we have previously demonstrated that LN-induced changes in p75NTR coincide with decreased Rho activity (Rankin et al. 2008b), I assessed the activation of Rho in response to a LN substrate in wild type PC12 cells, or Y490F and Y785F mutant cell lines which demonstrate constitutively low or high levels of PTEN, respectively. While wildtype PC12 cells and Y785F mutants responded to a LN substrate by decreasing Rho activity, the Y490F mutants did not (Figure 8.4). Wildtype PC12 cells, Y490F and Y785F TrkA mutants can be differentiated into a sympathetic neuronal phenotype and respond to NGF by extension of neuritic processes (Greene and Tischler, 1976). Since Rho is a potent inhibitor of neurite extension, and these three cell lines exhibit differential abilities to decrease Rho activity in response to a LN substrate, I compared the neurite growth response of NGF-differentiated cells following plating on LN. Growth quantification analyses revealed significantly reduced neurite growth in Y490F TrkA mutants plated on LN versus the parental PC12 cells (Figure 8.5A). In contrast, Y785F TrkA mutants demonstrated significantly enhanced growth in comparison with the parental line (Figure

**Figure 8.4: LN fails to induce depressed Rho activity in PC12 cell lines expressing a mutated Y490F TrkA receptor.** Parental PC12 cells, or PC12 derivative cell lines expressing either TrkA Y490F or Y785F mutations were subcultured on PL or LN substrates for 24 h prior to assessment of active Rho using a pulldown assay. GTP $\gamma$ S-loaded samples served as a positive control while GDP-loaded samples were utilized as a negative control. Values expressed represent the mean of 3 experiments  $\pm$  SEM where  $*p < 0.001$  as determined by one way ANOVA.

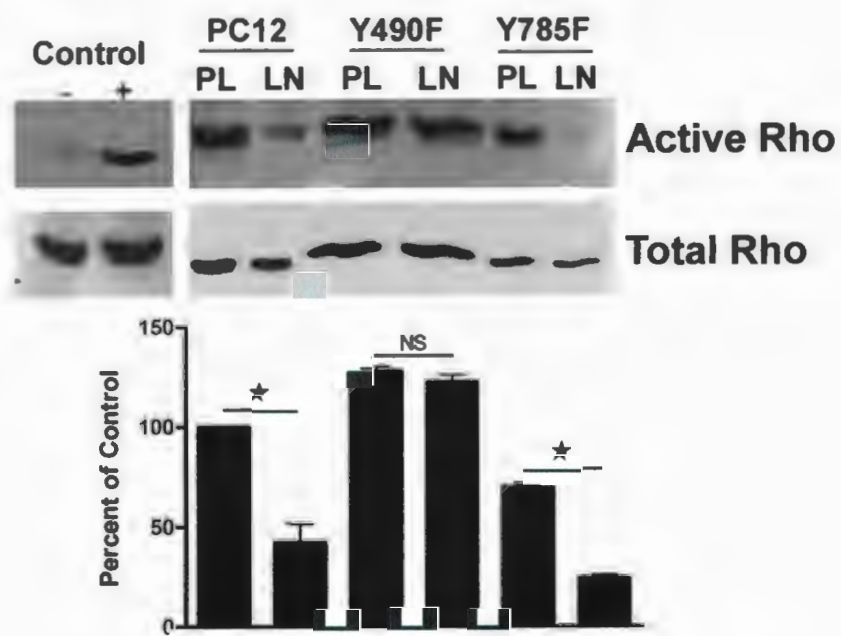
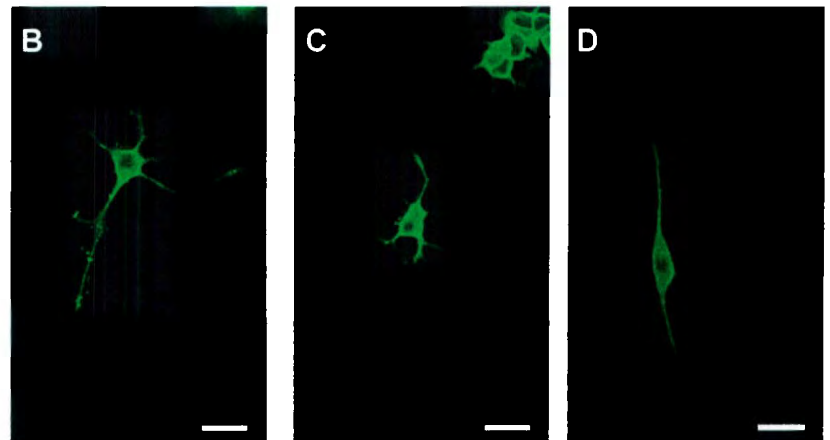
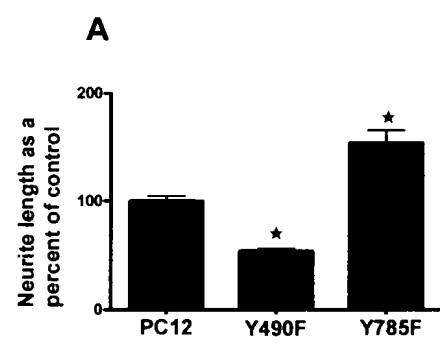


Figure 8.4

**Figure 8.5: Growth response to LN is altered in TrkA mutant PC12 cells.** NGF-differentiated PC12 cells, or PC12 derivative cell lines expressing either TrkA Y490F or Y785F mutations were subcultured on LN for 24 h prior to immunostaining with antibodies directed against total tubulin and growth assessment using image J software and a total analysis of 250 cells per condition (A). Relative to parental PC12 cells, Y490F cells exhibit an impaired growth response and Y785F cells show enhanced growth. Representative images of cell growth are depicted for PC12 cells (B), Y490F (C) and Y785F (D) TrkA mutants. Differences in neurite length (A) were considered significant at  $*p < 0.05$  as determined by one way ANOVA. Scale bar = 25 $\mu$ m.





**Figure 8.5**

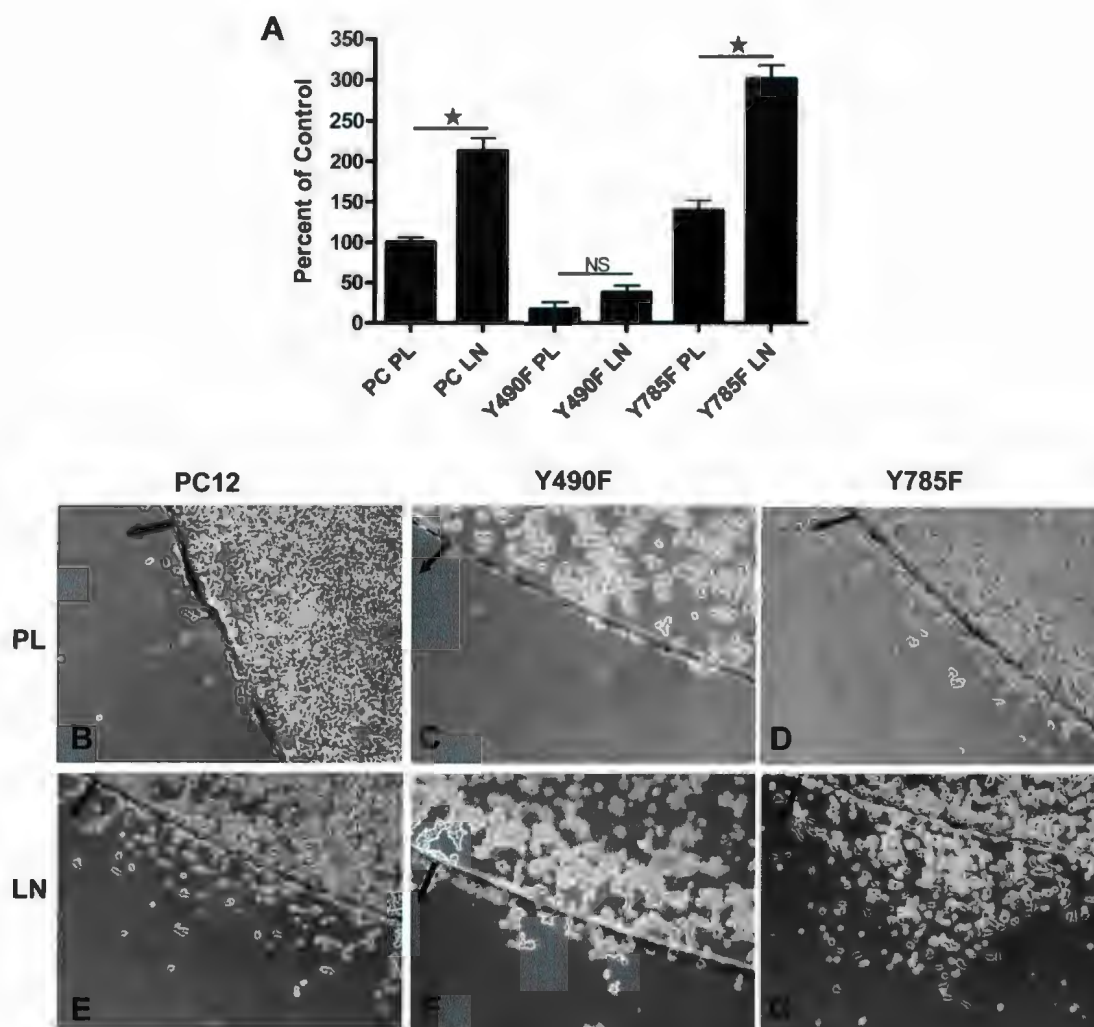
8.5A). Representative images of the growth response of each cell line to a LN substrate are depicted in Figure 8.5B-5D. These results suggest that TrkA regulation of PTEN and the subsequent impact on a LN-induced p75NTR-Rho signalling cascade alters the growth potential of differentiated PC12 cells when exposed to LN in the absence of NGF.

Rho activity and its regulation of the cytoskeleton also play a key role in cell migration (Raftopoulou and Hall, 2004). Since naive PC12 cells are motile in nature, I tested whether TrkA mutation regulation of PTEN and subsequent modulation of p75NTR and Rho activity, plays a role in the motile response of these cells to LN. Using our recently reported cell motility assay (Rankin et al., 2006), we examined the LN-induced motility of wildtype PC12 cells, or the Y490F and Y785F TrkA mutants show low or high expression of PTEN, respectively (see Figure 8.2). Y490F TrkA mutants were observed to exhibit significantly decreased migratory behaviour on a LN substrate, relative to wildtype PC12 cells (Figure 8.6A), while Y785F TrkA mutants are highly motile, in agreement with previous results (Rankin et al., 2006). Representative images of cell migration are depicted for wildtype PC12 cells (Figure 8.6B, 8.6C), Y490F (Figure 8.6D, 8.6E) and Y785F TrkA mutants (Figure 8.6F, 8.6G). These results suggest that in addition to altering the neurite growth potential of PC12 cells, TrkA may also influence the motile behaviour of these cells in response to a LN substrate.

#### **8.4 Discussion**

PTEN, a phosphatase best known as a tumour suppressor and for its well characterized antagonism of PI3K signal transduction, is emerging as a key player in the

**Figure 8.6: Motile response to LN is altered in TrkA mutant PC12 cells.** PC12 cells or PC12 derivative cell lines expressing either TrkA Y490F or Y785F mutations were cultured on PL-coated coverslips prior to inversion onto a LN substrate. Motility was assessed following 36 h culture wherein the number of motile cells was enumerated using 40 images per coverslip, with 3 coverslips per cell line in a total of 3 independent experiments (A). Y490F mutant cells exhibit significantly reduced motility in comparison with wt PC12 cells, while Y785F cells are significantly more motile than control PC12 cells. Significant differences were determined using one way ANOVA where  $*p < 0.05$ . Representative images of cell motility are depicted for PC12 cells (B, E), and TrkA mutants Y490F (C, F) and Y785F (D, G). Arrows indicate the direction of cell movement.



**Figure 8.6**



manipulation of the cytoskeleton for neuronal growth and motility. This study integrates PTEN's role in the growth response within the context of previously recognized components TrkA, p75NTR and Rho. Binding of individual Trk family members with their cognate ligand is well known to promote growth and migratory responses in the developing nervous system based on a well characterized series of signalling cascades (Reichardt, 2006). We have previously shown that neurotrophin stimulation via Trk and/or p75NTR does not affect PTEN expression by 24 h (Rankin et al. 2008b). I now report that constitutive expression of TrkA itself does impact the basal level of PTEN expression, likely over longer time periods. In fact, the presence of particular autophosphorylation sites within the cytoplasmic tail of TrkA differentially regulates PTEN expression in a manner that is inverse to p75NTR expression alterations previously reported (Rankin et al., 2005). This regulation may in fact be due to the low level of constitutive activation of the overexpressed Trk receptors, raising the possibility that this low level stimulation produces maximal effects on PTEN expression, which cannot be further enhanced by ligand binding. This phenomenon is noted in cerebellar granule neurons where a low level constitutive activation of TrkC is thought to prevent further stimulation by NT-3 (Zirrgiebel and Lindholm, 1996), thereby preventing the further activation of specific signalling responses. Alternatively, the receptor transactivation in the absence of ligand may be initiating different signalling than NGF-induced activation.

As TrkA regulates constitutive p75NTR expression, and we have reported that PTEN can regulate p75NTR expression (Rankin et al, 2008b), this raises the possibility that the modulation of constitutive p75NTR by TrkA is actually occurring via intermediate PTEN. Ligand binding to TrkA causes receptor dimerization resulting in the

transphosphorylation of two tyrosine residues outside of the kinase domain.

Phosphorylation of Y490 facilitates interaction with Shc, an adapter protein which regulates the Ras/MAP kinase pathway for growth and differentiation. Phosphorylation of Y785 recruits PLC $\gamma$  to activate the PKC/MAP kinase pathway which similarly influences cell growth. Additionally, activation of the Trk receptor is associated with induction of the PI3K/Akt pathway for neuronal survival. The fact that abrogation of either one, or both of these phosphorylation sites can manipulate PTEN expression levels provides preliminary evidence regarding the cell signalling pathways which may regulate PTEN, an area currently under investigation, though it appears from this mutational analysis that the lack of PLC $\gamma$  signalling increases PTEN expression, while a lack of Ras/MAP kinase activation decreases PTEN expression. This is consistent with our previous results that loss of PLC $\gamma$  signalling results in reduced p75<sup>NTR</sup> expression (Rankin et al., 2008).

The role of TrkA in the promotion of both growth and motility (Ho et al., 2001; Ho et al., 2005) is a well-known phenomenon, and in PC12 cells, this NGF-induced response appears to be dependent upon the phosphorylation of ERK, which is accomplished by phosphorylation of either Y490 or Y785, two pathways that seem to compensate for each other in this regard. However, despite similar NGF-stimulation-induced phosphorylation of ERK in the cells expressing TrkA Y490F and Y785F (data not shown), they demonstrate variable amplitude of response in terms of both growth and motility, indicating that the ECM is able to provide modulatory signals to amplify the response and adjust biological outcomes.



We have previously reported that in the absence of neurotrophins, a LN substrate is sufficient to promote neurite outgrowth of PC12 cells and hippocampal neurons (Rankin et al. 2008b), and cell migration of cerebellar granule neurons (Rankin et al. Manuscript submitted), by downregulating p75NTR and subsequently suppressing Rho activity. This cascade requires PTEN as a signalling intermediate (Rankin et al. 2008b). Here I demonstrate that the absence of PTEN associated with the Y490F TrkA mutation renders the cells incapable of modulating p75NTR in response to LN, and these cells subsequently fail to decrease Rho activity in response to LN substrate, leading to impaired growth and motility. The extension of neurites does occur in the absence of detectible PTEN protein expression, but to a lesser degree, while motility is severely limited. These findings highlight the importance of PTEN expression in facilitating LN-induced modulation of signal transduction cascades for remodelling of the actin cytoskeleton, likely via alterations in Rho activity.

The results of the current study suggest that TrkA modulates PTEN expression. This significantly contributes to the cells ability to respond to a LN substrate, which could impact the development or regenerative capacity of the nervous system. PTEN and p75NTR dysregulation have also been independently associated with a wide variety of diseases (Dechant and Barde, 2002; Schor, 2005). Therefore determination of their regulatory cascades is of key importance to further our understanding of both how pathological conditions may arise and how they may be therapeutically targeted.

## **Chapter 9: The rapid upregulation of PTEN following integrin ligation is dependent upon focal adhesion signalling through Egr-1.**

### **9.0 Summary**

Extracellular matrix signals are mediated by integrins, receptors that link extracellular signals to the actin cytoskeleton and activate signalling cascades to regulate axonal outgrowth and motility. We have recently reported that integrin ligation by laminin (LN), an extracellular matrix component, can induce upregulation of PTEN, a phosphatase, thus initiating a signalling cascade for the promotion of growth and migration. I now investigate the mechanism by which LN upregulates PTEN through a series of experiments designed to address the temporal and dose-dependent response to LN, and to ascertain the contribution of integrin-associated intermediates to this cascade. I demonstrate that integrin activation induces the upregulation of PTEN very quickly and at low concentrations of the LN stimulus, and in a variety of cell lines including PC12 cells and the human hepatoma-derived cell lines HepG2 and Huh7. This signalling paradigm occurs via a pathway involving focal adhesion components Src, ILK and FAK, as inhibition of these components decreases PTEN induction in response to LN exposure. Furthermore, I report a role for transcription factor Egr-1, which is upregulated by LN exposure; the selective decrease of Egr-1 using siRNA impairs the upregulation of PTEN in response to LN. These results provide details of a novel signalling mechanism which may influence neurite outgrowth and motility.



## 9.1 Introduction

The three-dimensional extracellular environment of a cell is comprised of the extracellular matrix (ECM), which itself is composed of a variety of proteins (e.g. laminin, fibronectin) synthesized and secreted by resident cells. During development, the ECM serves as a substrate for axonal growth, and subtle differences in its composition can effectively guide axons long distances to appropriate targets (reviewed in Luckenbill-Edds, 1997). The ECM persists into adulthood, and differs between the peripheral and central nervous system thereby contributing to differences in the regenerative growth response of PNS or CNS neurons (reviewed in Filbin, 2006).

ECM molecules interact with a group of receptors known as integrins. Integrins are  $\alpha/\beta$  heterodimers, wherein specific combinations recognize and bind particular molecules of the ECM. Ligand binding results in integrin aggregation within the cell membrane and in subsequent recruitment of a variety of different adapter proteins and kinases forming focal adhesions. It is through these focal adhesions that integrin signalling can initiate a variety of downstream signal transduction cascades to alter gene transcription to regulate the actin cytoskeleton for a variety of physiological outcomes, including neurite outgrowth and motility (Reviewed in Lemons and Condic, 2008).

We have recently presented evidence that laminin (LN), a common component of the peripheral ECM with known growth-promoting abilities, can act via integrin receptors to upregulate the expression of PTEN, thus initiating a signalling cascade that promotes neurite outgrowth in the absence of neurotrophins (Rankin et al. 2008b). PTEN is a phosphatase, best known for its role as a tumour suppressor and its antagonism of the PI3K survival pathway, and is thus under tight regulatory control. PTEN is, however, a

dual specificity phosphatase able to dephosphorylate both lipid and proteins substrates (Salmena et al., 2008). New functions in growth and motility are beginning to emerge for the protein phosphatase abilities of this enzyme (Leslie et al., 2007). We have previously demonstrated that the protein phosphatase activity of PTEN decreases the DNA binding ability of transcription factor Sp1 in cells exposed to LN, thereby decreasing p75NTR transcription and subsequent protein expression (Rankin et al. manuscript submitted). This cascade results in enhanced axonal regeneration in differentiated PC12 cells and developing hippocampal neurons (Rankin et al. 2008b), as well as increased motility of cerebellar granule neurons (Rankin et al. manuscript submitted) by Rho-induced modulation of the actin cytoskeleton.

The present study investigates the mechanism by which LN results in the upregulation of PTEN via downstream activation of integrin-associated signalling intermediates. My results show that ECM molecules act via integrin focal adhesion components and the transcription factor Egr-1 to upregulate PTEN. This regulation may play a role in determining survival and axonal growth in response to the ECM in the developing nervous system and during the regenerative growth response promoted by exposure to LN.

## **9.2 Materials and Methods**

### **9.2.1 Cell Culture and Differentiation**

The experimental model consisted of wild-type rat pheochromocytoma (PC12) cells, and a series of mutated PC12 derivative cell lines (gifts from Dr. David Kaplan, Hospital for Sick Children, Toronto, ON). PC12nnr5 cells were derived by mutating parental PC12 cells using ethylmethanesulfonate, as described by Green et al. (1986). PC12 derivatives expressing mutant TrkA receptors (Y490F or Y785F) were created by Stephens et al. (1994). Briefly, PC12nnr5 cells were transfected to express a TrkA receptor that had been altered via site-directed mutagenesis to abrogate either of the autophosphorylation sites of the cytoplasmic tail.

Cell lines were maintained on rat-tail collagen-coated tissue-culture flasks in RPMI 1640 medium (Invitrogen, Burlington ON) supplemented with 10% horse serum (Invitrogen), 5% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin/glutamine solution (PSG; Invitrogen) as previously described (Rankin et al., 2005; Rankin et al., 2008; Rankin et al., 2006). In the case of mutated cell lines Y490F and Y785F, media was additionally supplemented with Geneticin G418 to ensure continued selection of the mutant population. Cells were incubated at 37 °C in 5% CO<sub>2</sub>, and cultured to 80% confluence prior to trypsinization for subculturing and differentiation.

HepG2 and Huh7 human hepatoma-derived cells were maintained in DMEM containing 10% fetal calf serum and 1% penicillin/streptomycin/glutamine solution.



### **9.2.2 Primary Cell Culture**

Cultures enriched in granule neurons were obtained from cerebella of 8 day-old Sprague-Dawley rat pups as described previously (Jiang et al., 2003; Rankin et al., 2008). Cerebella were stripped of meninges, finely chopped and dissociated by enzymatic digestion using 0.25% trypsin-EDTA followed by mechanical trituration. The culture medium was Dulbecco's modified eagle medium (DMEM) supplemented with 10% FCS, 25 mM KCl and 1% penicillin/streptomycin solution (Invitrogen). Cells were seeded onto polylysine (PL; 40 µg/ml) or laminin (LN; 25 µg/ml)-coated tissue culture plates at a density of  $1.8 \times 10^6$  cells per ml. Cells were incubated at 37°C in 5% CO<sub>2</sub> and cytosine arabinoside was added to longterm cultures to inhibit the proliferation of non-neuronal constituents. On the seventh day in vitro (DIV) of longterm cultures, glucose (50 µl of a 100 mM solution) was added to each culture well to maintain survival, as culture medium was not replaced. This method yields cultures consisting of approximately 95% CGN (Jiang et al., 2003).

### **9.2.3 Constructs and Transfection**

HA-FRNK plasmid DNA was a gift from Dr. K. Vuori, Burnham Institute, La Jolla, CA. Plasmids encoding ILK wildtype, kinase dead ILK (R211A) and dominant negative ILK (E359K) were a gift from Dr. Daniel MacPhee (Memorial University of Newfoundland). Vectors were introduced to the differentiated PC12 cells using Lipofectamine 2000 as per the manufacturer's instructions.



#### **9.2.4 Inhibition Studies**

For pharmacological inhibition studies, cells were similarly subcultured on PL-coated 12-well plates under serum-starved conditions. The pharmacological inhibitor, PP2 (various concentrations as indicated; Calbiochem) were separately added to individual wells at the time of plating. Inhibitor concentrations were empirically determined to provide the required inhibition (Fig 3A, 3D, 4C) with no detriment to viability (Dodge et al., 2002; Mearow et al., 2002). Cells were then allowed to incubate for 24 h at 37°C in 5% CO<sub>2</sub>.

#### **9.2.5 Western Blot Analysis**

For Western analyses, cells were harvested in the presence of sodium orthovanadate and subsequently subjected to lysis (10% glycerol, 1% NP-40, sodium vanadate, sodium fluoride, magnesium chloride, octyl- $\beta$ -thioglucopyranoside (Sigma), and 1 protease inhibitor cocktail tablet (Roche Scientific, Laval, QC) overnight at 4° C prior to centrifugation (10,000 rpm, 5 minutes). A BCA protein assay (Pierce, Rockford IL) was used to determine protein concentration and equivalent amounts of protein (50  $\mu$ g) were electrophoresed on 8% SDS-polyacrylamide gels. Protein was subsequently transferred to nitrocellulose membranes that were then exposed to Ponceau red to ensure equal protein loading. After washing in TBS, blots were blocked in 3% non-fat dry milk for 1 h at room temperature, and then incubated with a primary antibody overnight at 4° C. Antibodies used include: Anti-Actin (Sigma), Anti-PTEN (Cell Signaling). A final incubation with HRP-conjugated secondary antibody (AP307P, AP308P; Chemicon,) for

1 h at room temperature was followed by visualization using an ECL method. Each experiment was repeated three times, subjected to densitometry and normalized to actin to ensure equal protein for comparison. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego CA) with significance being determined using one-way ANOVA testing.

#### **9.2.6 Real-time RT-PCR**

For real time RT-PCR analysis, cells were subcultured on PL or LN-coated 12-well plates under serum-starved conditions for 24 h in the presence or absence of NGF (50 ng/ml). RNA was isolated using Trizol reagent (Invitrogen) as per the manufacturer's instructions and was subsequently treated with DNase (Ambion; Austin TX) to remove traces of contaminating DNA. RNA (2 µg) was reverse transcribed to cDNA using MMLV reverse transcriptase (200 U for 30 minutes at 37 °C; Invitrogen) prior to use as template for real-time PCR amplification using the following PCR primer pairs: PTEN forward 5'-ATTCGACTTAGACTTGACCT, PTEN reverse 5'-ACCAGTCCGTCCTTTC, 28S forward 5'-GACCAAGGAGTCTAACGC, 28S reverse 5'-GTACGCTCGTGCTCCA, Egr-1 forward 5'-CAGGAGTGATGAACGCAAGA, Egr-1 reverse 5'-AGCCCGGAGAGGAGTAAGAG. PCR amplification was performed using the Roche LightCycler (Roche) and quantified using SYBR green I. Egr-1 and PTEN mRNA expression levels were subsequently normalized to 28S.

### **9.2.7 Small interfering RNA constructs and transfection**

Egr-1 siRNA constructs were synthesized by Dharmacon as follows: target sequence 5'-GCGAACAACCCUACGAGCA. PC12 cells were transfected with 1  $\mu$ M of Egr-1 siRNA using Dharmafect transfection reagent according to manufacturer's protocols. The siRNA transfection efficiency was assessed to be >70% based on transfection of Alexa fluor-labelled negative control siRNA (scrambled; target sequence AATTCTCCGAACGTGTCACGT; Qiagen, Mississauga ON). 24-48 hours post-transfection, cells were transferred onto PL or LN coated tissue culture plates for protein analysis after an additional 24 hours.

## **9.3 Results**

### **9.3.1 LN is a potent upregulator of PTEN expression**

We have previously determined that exposure of PC12 cells to a laminin substrate upregulated PTEN mRNA and protein expression, and downregulated p75NTR expression (Rankin et al 2008b). To ascertain whether there existed a dose-dependent relationship between integrin activation and modulation of PTEN, PC12 cells and cerebellar granule neurons were plated on substrates coated with varying LN concentrations (Figure 9.1). The results show that the minimum concentration of LN utilized, (1  $\mu$ g/ml), was sufficient to induce an upregulation of PTEN as assessed by Western blotting in both PC12 cells (Figure 9.1A) and CGN (Figure 9.1C). The expression of p75NTR changes inversely in each cell type, downregulating in response to



**Figure 9.1: PTEN expression is induced by low concentrations of LN.** PC12 cells (A,B) or CGN (C,D) were plated on varying concentrations of LN as indicated and stimulated for 24 h prior to analyses by Western blotting. The lowest concentration of LN tested (1µg/ml) elicited a significant increase in PTEN expression (A,C) which remained elevated throughout the range of concentrations evaluated. Exposure to all tested LN concentrations was also associated with a significant downregulation of p75NTR (B,D). Values represent the mean +/- SEM expression level following normalization against actin, obtained from 3 independent experiments, where \* $p < 0.05$  as determined by one way ANOVA. Values are expressed as a percentage of the control PL condition.



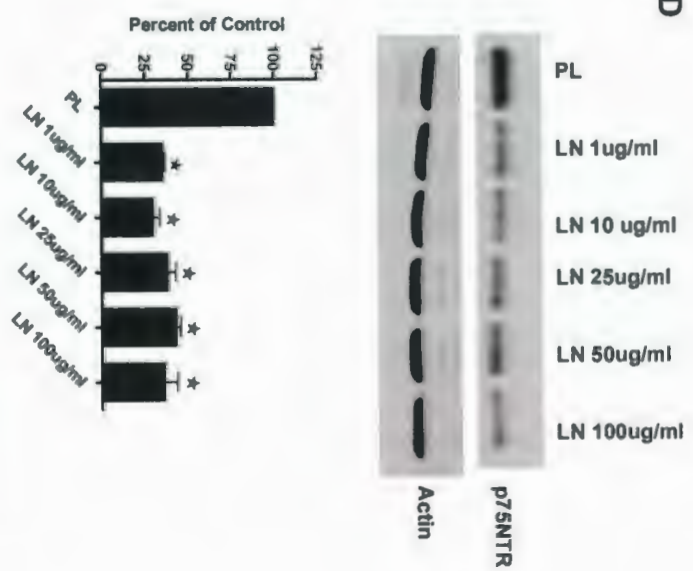
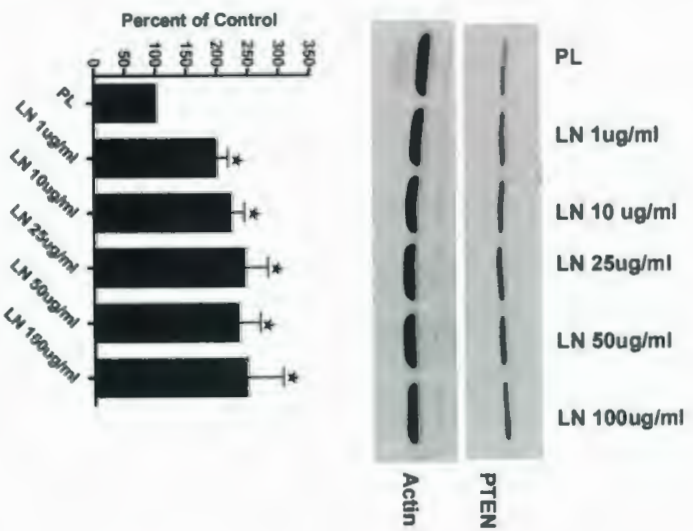
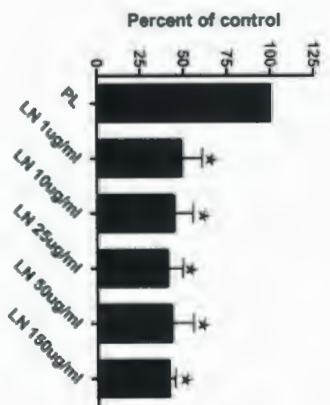
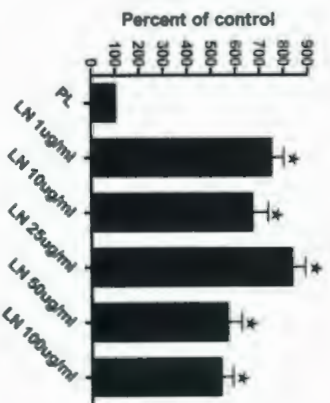
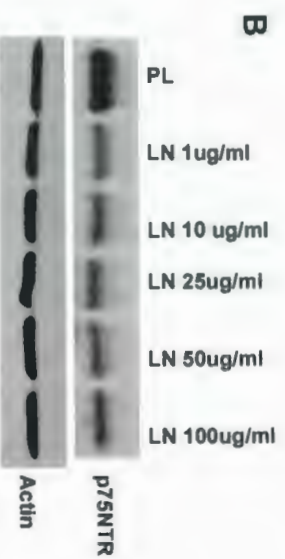
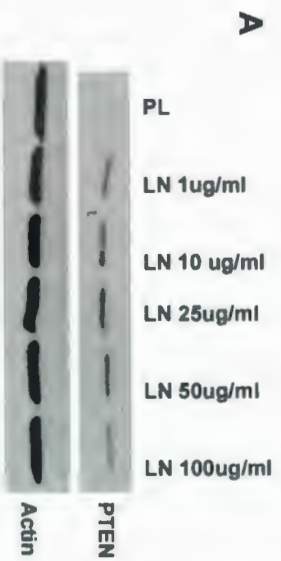


Figure 9.1

each LN concentration (Figure 9.1B, D). Interestingly, the increase in PTEN expression and decrease in p75NTR expression was maximal upon stimulation with 1  $\mu\text{g/ml}$  LN, and failed to demonstrate a dose-dependent response to LN when utilized at concentrations up to 100  $\mu\text{g/ml}$ .

### **9.3.2 Exposure to a LN substrate rapidly modulates PTEN expression**

Since integrin activation was observed to modulate PTEN expression (Figure 9.1), we assessed the kinetics of the LN-mediated upregulation of PTEN in cultured PC12 cells or a mutated derivative, PC12nnr5. PC12nnr5 cells lack TrkA, but express a significantly greater constitutive level of PTEN expression. Cells were subcultured and plated on either PL or LN substrates for culture periods varying between 1 to 24 h as indicated in Figure 9.2, prior to protein collection and analysis by Western blotting. The results indicate that exposure to the LN substrate results in significant upregulation of PTEN protein in both PC12 (Figure 9.2A) and PC12nnr5 cells (Figure 9.2C) as early as 1 h post-LN exposure, while significant differences in p75NTR expression between the PL and LN conditions were not apparent until 6 h in both PC12 cells (Figure 9.2B) and PC12nnr5 cells (Figure 9.2D). To confirm that this increased expression of PTEN was actually transcriptional in nature, I performed real-time RT-PCR analysis for PTEN expression following timed intervals of LN exposure from 1-24 h as indicated (Figure 9.2E). Elevated levels of PTEN mRNA are evident as early as 1 h post-LN exposure, and persisted throughout the first 24 h in culture, in agreement with our previously reported results (Rankin et al. 2008b). p75NTR mRNA expression was suppressed by 6 h following exposure to the LN substrate (Figure 9.2F). Taken together,

**Figure 9.2: LN stimulation modulates PTEN gene transcription and protein expression as early as 1 h post stimulation.** PC12 (A, B, E, F) and PC12nnr5 (C, D) cells were plated on a LN substrate and analyzed for changes in PTEN protein (A, C) and mRNA (E) or p75NTR protein (B, D) and mRNA (F) expression using Western blot and real-time RT-PCR analyses, respectively. PTEN protein expression was significantly elevated as early as 1 h post-stimulation (A, C), co-incident with increased PTEN mRNA transcription (E). Downregulation of p75NTR protein (B, D) and mRNA (F) could be detected by 6 h following exposure to the LN substrate. Protein levels were normalized against actin, while cDNA samples were normalized using 28s. Values represent mean expression levels  $\pm$  SEM obtained from three independent experiments, and are presented relative to the control PL condition where  $*p < 0.05$  as determined by one way ANOVA. Values are expressed as a percentage of control condition PL 1h (A-D) or time 0 (E-F).



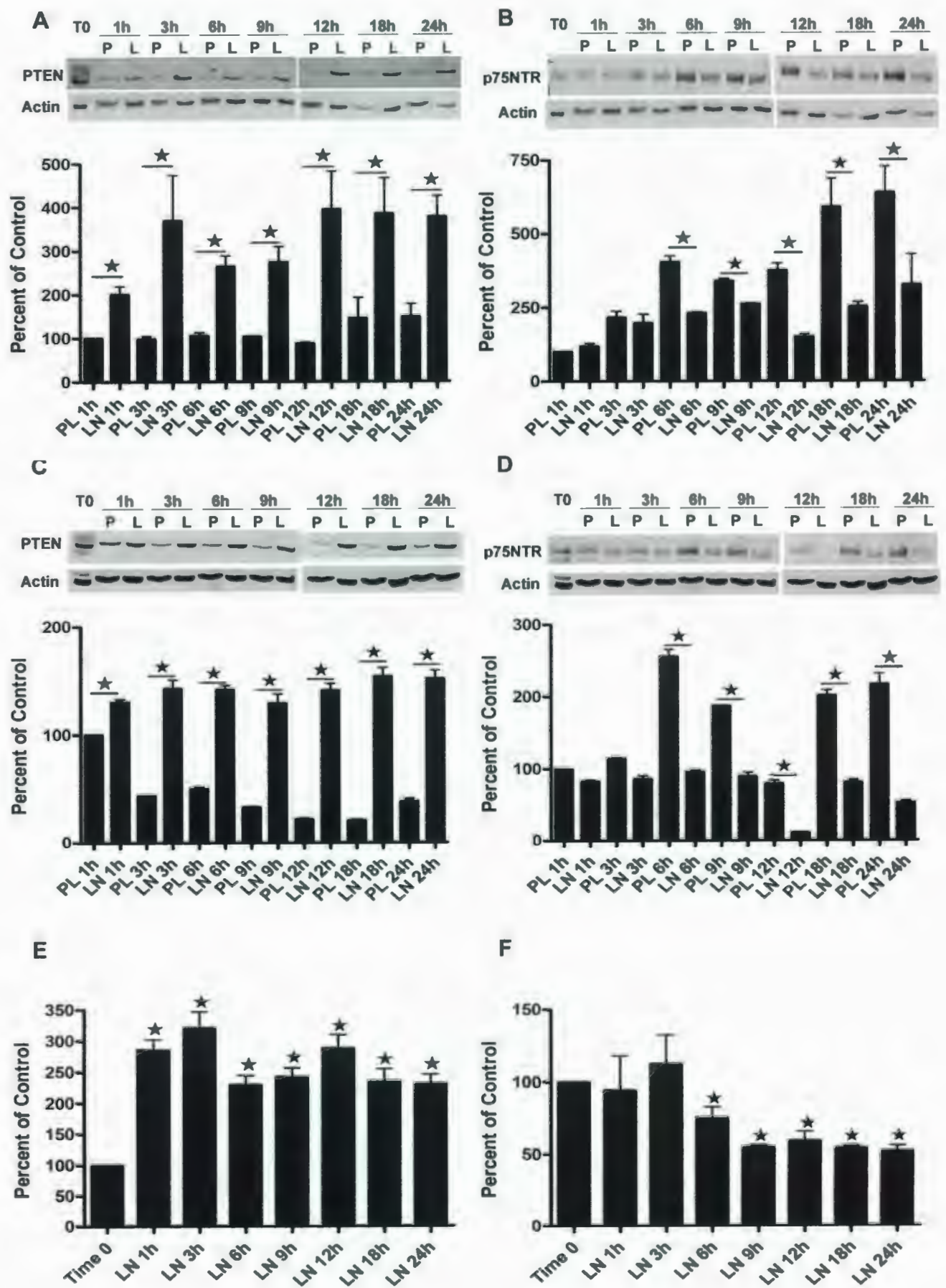


Figure 9.2



my results suggest that LN may potentially induce PTEN expression levels both in terms of the minimal threshold of integrin activation required (Figure 9.1), and in the expediency of this signalling paradigm (Figure 9.2). Furthermore, p75NTR expression is consistently inverse, but is subject to slower expression changes, suggesting that PTEN changes are upstream of changes to p75NTR.

### **9.3.3 Regulation of PTEN expression following integrin stimulation is not restricted to a single cell type or to exposure to a LN substrate.**

PC12 cells are maintained on a collagen substrate which itself imparts integrin activation. Thus, while the LN substrate was a potent inducer of PTEN expression in these cells relative to PL, I confirmed whether integrin activation may modulate PTEN expression in cell lines which are maintained in the absence of exogenous integrin stimulation. Thus, human hepatoma cell lines HepG2 and Huh7 which are normally maintained on tissue culture plastic in the absence of exogenous integrin activating substrates, were plated on PL or LN substrates for 24 h prior to analysis of PTEN protein expression by Western blotting. The results confirm that these two cell lines also respond to the LN substrate with a significant upregulation of PTEN (Figure 9.3). Thus, integrin activation is sufficient to activate this LN-induced signalling response in multiple cell types.

Integrin activation is not strictly a function of LN stimulation, but rather specific  $\alpha\beta$  heterodimers respond to particular components of the ECM. However, integrin clustering and initiation of downstream signalling pathways is largely conserved, with subtle differences and proceeds via formation of focal adhesions and recruitment of

**Figure 9.3: LN mediates changes in PTEN expression in cells which are not of neuronal lineage.** Human hepatoma-derived HepG2 and Huh7 cells were plated on PL or LN substrates for 24 h prior to Western blot analysis of PTEN expression. LN induced significant upregulation of PTEN expression relative to the control PL condition. Values represent mean expression levels following normalization to actin, and are presented as a percentage of the control PL condition, +/- SEM, where \* $p < 0.05$  as determined by one way ANOVA.

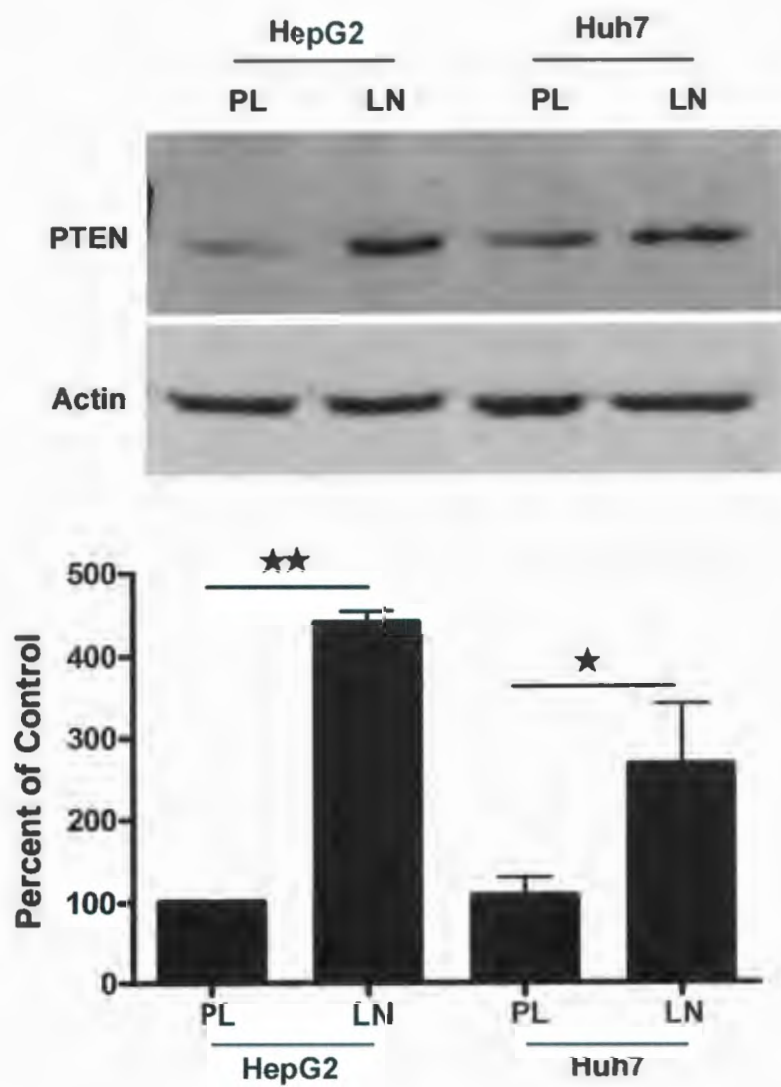


Figure 9.3

common signalling adaptor proteins and kinases (Coppolino and Dedhar, 2000; Lemons and Condic, 2008). To examine whether integrin activation and signalling in general, was sufficient to initiate upregulation of PTEN expression, I utilized several integrin-activating substrates and assessed their capacity to modulate PTEN (Figure 9.4). Thus, PC12 cells or CGN were plated on PL, LN, matrigel (a synthetic basement membrane composed largely of LN), collagen (Col) and fibronectin (FN) for 24 h prior to Western blot analyses. Each of these substrates was associated with a significant upregulation of PTEN expression in PC12 cells (Figure 9.4A), and all but collagen resulted in significant upregulation of PTEN in CGN (Figure 9.4B). Thus, integrin regulation of PTEN extends beyond the LN substrate, and likely includes any integrin activating substrate for which the cell expresses the appropriate integrin pair.

#### **9.3.4 Inhibition of integrin-associated signalling components impairs PTEN expression**

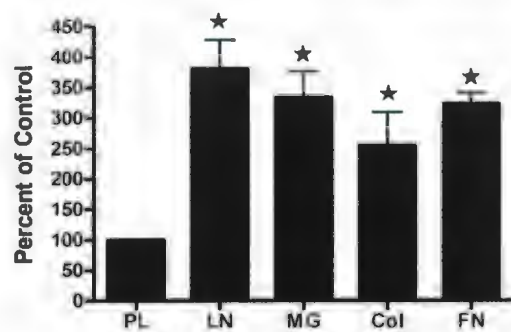
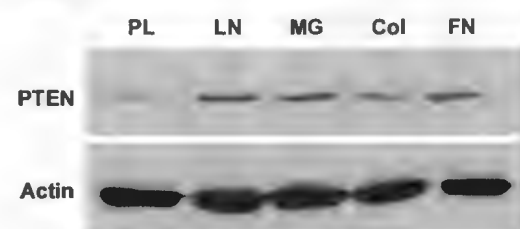
Intramembrane clustering of integrin receptors in response to ligand binding results in recruitment of a variety of kinases, including Src family kinases, focal adhesion kinase (FAK) and integrin-linked kinase (ILK) and adapter molecules which subsequently initiate signalling cascades to shape cellular behaviour. To investigate whether integrin-associated kinases, recruited to the focal adhesions, are necessary for PTEN regulation, we utilized pharmacological or genetic inhibition of these common protein kinases. When cultured on a PL substrate, the basal activation of integrin associated signalling components is quite low in general, but dramatically increases when the cells are exposed to LN. Treatment of PC12 cells with the Src inhibitor PP2 for 24 h inhibits the



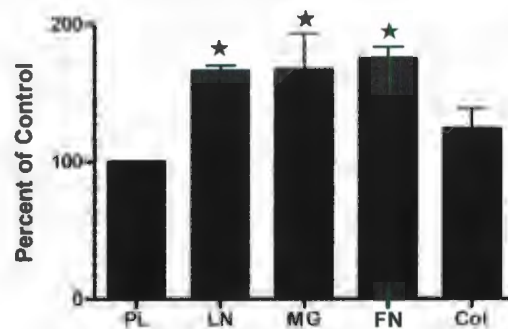
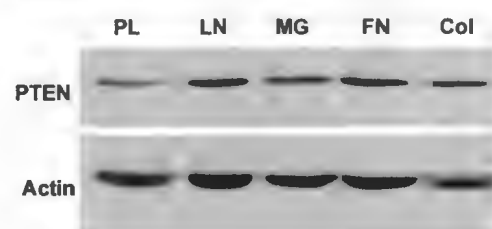
**Figure 9.4: LN-independent integrin activation is sufficient to alter PTEN**

**expression.** PC12 cells (A) or CGN (B) were plated on control PL, or integrin-activating substrates LN, matrigel, collagen or fibronectin for 24 h prior to Western blot analyses for PTEN expression. Each of the substrates was associated with an upregulation of PTEN expression in PC12 cells (A), and all substrates with the exception of collagen were associated with an upregulation of PTEN expression in CGN (B). Values represent mean expression levels following normalization to actin, and are presented as a percentage of the control condition, PL, +/- SEM, where  $*p < 0.01$  as determined by one way ANOVA.

**A** PC12



**B** CGN



**Figure 9.4**

phosphorylation of Src and impaired the upregulation of PTEN expression normally induced by LN exposure, and appeared to act in a dose-dependent fashion (Figure 9.5). I further investigated the importance of focal adhesion signalling in mediating the LN-induced upregulation of PTEN by interfering with FAK, which is recruited to sites of focal adhesions following integrin ligation. For this purpose, we utilized plasmid DNA encoding FAK-related non-kinase (FRNK), a non-catalytic C-terminal region of FAK which serves to inhibit endogenous FAK phosphorylation and downstream signalling. 36 h following transient transfection in PC12 cells, PC12 cells were exposed to either a PL or LN substrate for 24 h. Western blot analyses showed that inhibition of FAK activity significantly reduced the upregulation of PTEN in response to LN (Figure 9.6) relative to an empty vector control. ILK is an additional kinase which interacts with the cytoplasmic tail of integrin  $\beta 1$  subunit following integrin ligation. To examine whether this component of integrin signalling also impairs LN-mediated induction of PTEN I utilized constructs encoding wild-type ILK, or the mutations rendering ILK kinase dead (R211A), or dominant negative (E359K). As shown in Figure 9.7, overexpression of E359K in PC12 cells significantly impairs the upregulation of PTEN in response to a 24 h LN exposure, while the wild-type and kinase dead constructs had no effect (Figure 9.7). Thus, interference with any of the key components of focal adhesions tested significantly impaired the capacity of LN to modulate PTEN levels.

**Figure 9.5: Inhibition of integrin-associated signalling molecule Src impairs PTEN upregulation in response to LN exposure.** PC12 cells were plated on LN in the presence or absence of varying concentrations of the Src inhibitor PP2 for 24 h, as indicated. Western blot analyses confirm a dose-dependent reliance upon Src activation for the LN-induced expression of PTEN. Values represent the mean expression values  $\pm$  SEM following normalization to actin, obtained in 3 independent experiments, and are presented as a percentage of the control condition in the absence of Src inhibition. Differences were considered significant where  $*p < 0.05$  as determined by one way ANOVA.



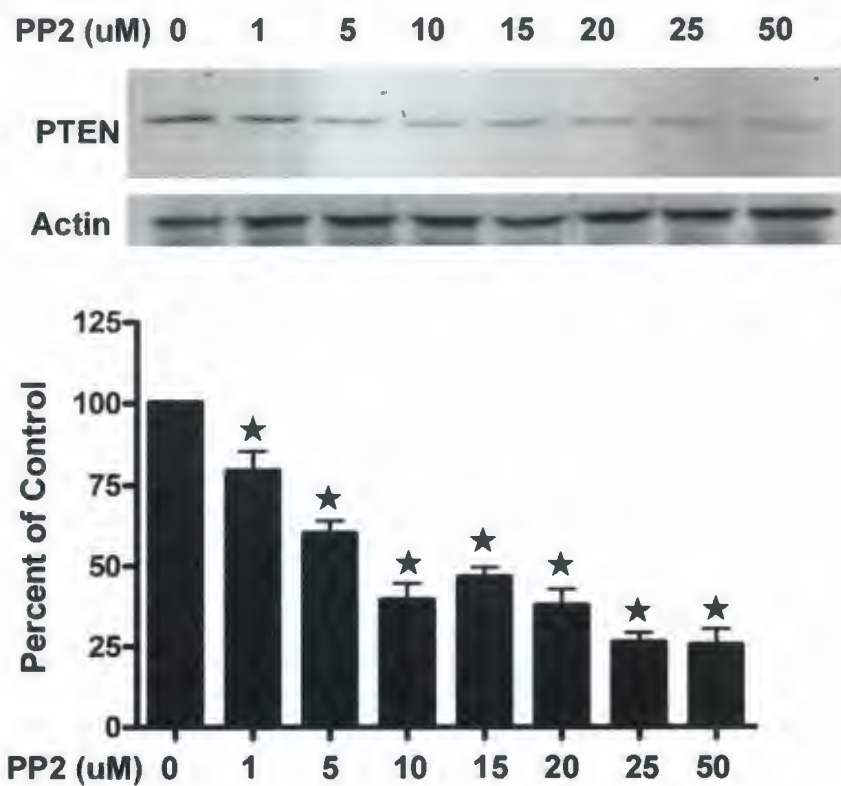


Figure 9.5

**Figure 9.6: Inhibition of focal adhesion kinase impairs LN-induced alterations in PTEN.** PC12 cells were transiently transfected with control empty vector, or vector encoding the FAK inhibitor FRNK, for 24 h prior to Western blot analyses. FRNK expression abrogated the LN-mediated upregulation of PTEN. Data represent the mean expression values obtained in 3 independent experiments following protein normalization to actin, and are presented as the mean  $\pm$  SEM relative to the empty vector control condition, where  $*p < 0.05$  as determined by one way ANOVA.

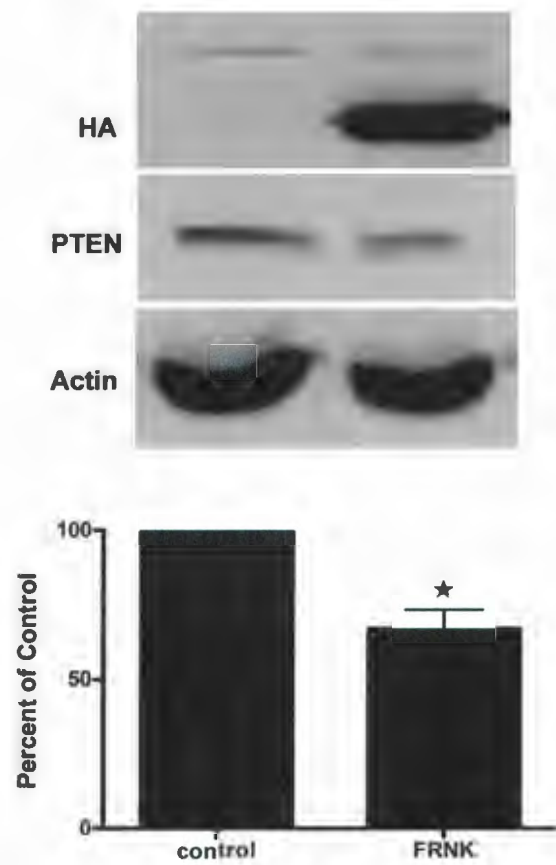


Figure 9.6

**Figure 9.7: Interference with integrin linked kinase impairs LN-induced alterations in PTEN.** PC12 cells were transiently transfected with control vector, or vectors encoding ILK wt, kinase dead ILK (R211A) or dominant negative ILK (E359K), prior to plating on LN substrates for 24 h. Dominant negative ILK construct (E359K) prevented the LN-induced upregulation of PTEN. Values are expressed relative to the control empty vector following protein normalization to actin, obtained in 3 independent experiments. Data represent the mean, +/- SEM, and changes in PTEN expression were considered significant when  $*p < 0.05$  as determined by one way ANOVA.



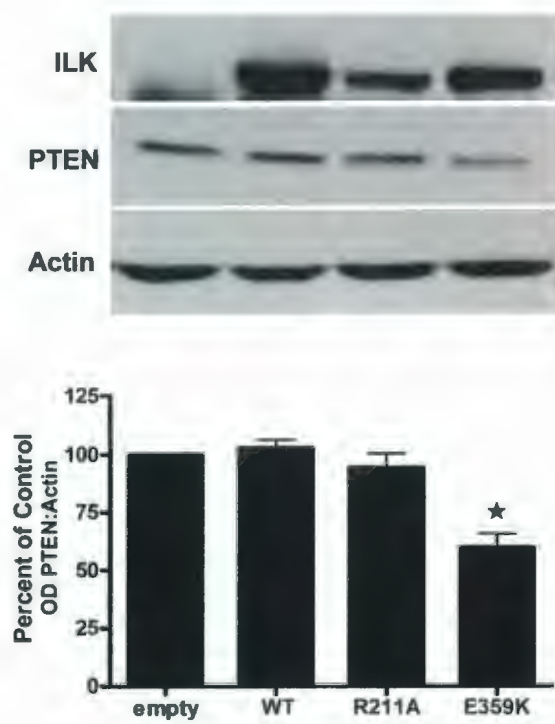


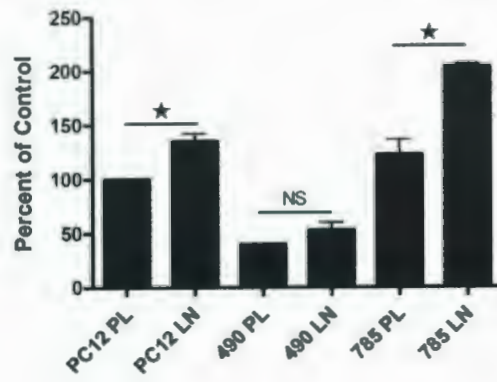
Figure 9.7

### **9.3.5 LN mediated regulation of PTEN expression requires the transcription factor, Egr-1.**

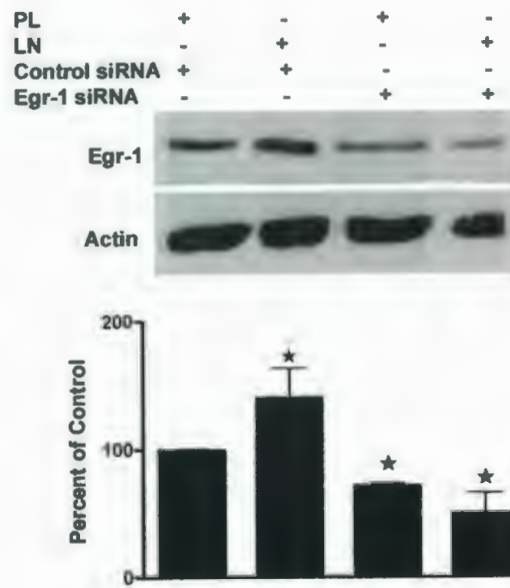
Previous reports have suggested that collagen stimulation results in an upregulation of the transcription factor Egr-1 (Barbolina et al., 2007), and Egr-1 is known to promote the transcriptional upregulation of PTEN in response to UV irradiation (Virolle et al., 2001). Furthermore, Egr-1 has been shown to be upregulated and translocated to the nucleus in vascular smooth muscle cells upon LN exposure (Morawietz et al., 1999), and LN induces the upregulation of Egr-1 in synovial fibroblasts (Warstat et al., 2008). Thus, I investigated whether exposure to a LN substrate modified the expression of Egr-1, as a potential mechanism regulating the observed LN-induced upregulation of PTEN. Semi-quantitative and real-time RT-PCR analyses confirmed that LN stimulation resulted in significant upregulation of Egr-1 mRNA in PC12 cells (Figure 9.8A). Interestingly, analyses of two PC12-derivative cell lines which express TrkA mutations, suggested that the Y490F mutants which constitutively express very low levels of PTEN and do not upregulate PTEN in response to LN (Rankin et al. Manuscript in preparation), express comparatively low levels of Egr-1 (Figure 9.8A). In contrast, Y785F mutants which constitutively express greater amounts of PTEN, and do respond to LN stimulation with increased PTEN expression (Rankin et al. Manuscript in preparation), transcribe significantly greater Egr-1 mRNA (Figure 9.8A). To determine if Egr-1 activity is associated with the LN-induced upregulation of PTEN, I utilized Egr-1 siRNA to reduce the expression of Egr-1 prior to exposing cells to either a PL or LN substrate. The Egr-1 siRNA significantly decreased the protein expression of Egr-1 (Figure 9.8B), and this selective abrogation of Egr-1 significantly impaired the

**Figure 9.8: Egr-1 transcription factor expression correlates with constitutive PTEN expression and its modulation in response to LN.** PC12 cells or derivative cell lines expressing TrkA mutations Y490F or Y785F, were plated on PL or LN substrates for 24 h prior to PTEN mRNA analysis using real-time RT-PCR (A). LN stimulation upregulated Egr-1 transcription in parental PC12 cells and the Y785F mutant, while Y490F mutants exhibited decreased constitutive expression of Egr-1 which was not influenced by LN stimulation (A). PC12 cells were transiently transfected with scrambled or Egr-1-specific siRNA prior to exposure to a LN substrate for 24 h (B, C). Western blot analyses confirm the successful downregulation of Egr-1 protein (B) and a requirement for Egr-1 in the LN-induced upregulation of PTEN protein expression. Data represent mean values obtained in 3 independent experiments, following cDNA normalization to 28s (A) or protein equalization to actin (B), and are presented relative to control PL (A) or scrambled (B) conditions. Differences were considered to be significant at  $*p<0.05$  as determined by one way ANOVA.

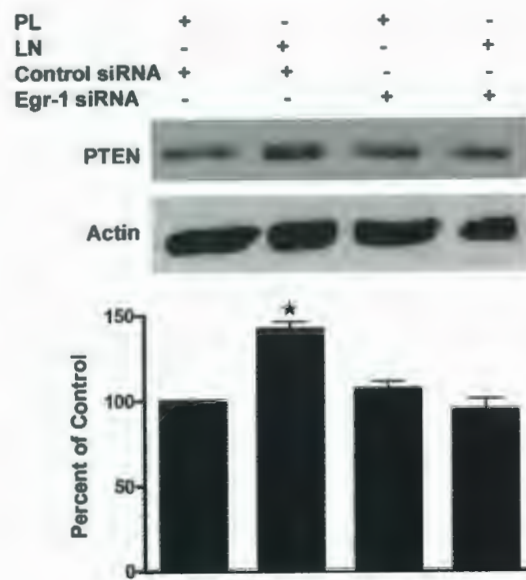
**A**



**B**



**C**



**Figure 9.8**



upregulation of PTEN in response to LN exposure (Figure 9.8C). Together, these results suggest an important role for Egr-1 both in the constitutive regulation of PTEN, and in regulating the induction of PTEN in response to integrin activation in PC12 cells.

#### **9.4 Discussion:**

This study explores a signalling mechanism initiated by ECM-integrin interaction which results in the upregulation of phosphatase, PTEN. I demonstrate that PTEN upregulation is a remarkably sensitive response that occurs at low concentrations of LN stimulus (1  $\mu\text{g/ml}$ ), and occurs very quickly with increased mRNA and protein evident within an hour of LN exposure. This rapid and sensitive induction of PTEN is dependent upon signalling through various focal adhesion components, including FAK, Src and ILK, and involves transcription factor Egr-1, an immediate early gene. Importantly, this early induction of PTEN occurs within an appropriate timeframe to induce other genetic changes, including the downregulation of p75<sup>NTR</sup>; a sequence of signalling events which we have previously shown to enhance axonal regeneration (Rankin et al. 2008b).

The ECM provides structural support, but can also regulate the biological responses of cells that it contacts by activating signalling cascades through integrin receptors, thus influencing critical developmental processes, including neuronal differentiation, migration and neurite outgrowth (Porcionatto, 2006). The ECM is of dynamic composition, as its individual components are temporally and spatially regulated throughout development, thus contributing to the growth and guidance of developing

neurons (Lemons and Condic, 2008; Porcionatto, 2006). Additionally, ECM components can be induced in the adult PNS following injury (Lemons and Condic, 2008).

The growth cone of an extending axon is able to readily adapt to and extend over the various different concentrations of LN it encounters as it extends through the developing embryo, from low levels in mesenchymal tissues to very high levels in basement membranes. This flexibility is necessary for accurate peripheral innervations (Lemons and Condic, 2006; Pietri et al., 2004), and may be attributable to the rapid modulation of receptor expression levels. The expression of integrin receptors are tightly regulated to match cellular requirements and maintain an intermediate level of attachment for efficient growth and motility (Condic and Letourneau, 1997), though forced expression of particular integrin subunits, and presumably enhancement of integrin-related signalling, can restore adult regenerative growth to embryonic levels (Condic, 2001). Injury, and the regenerative response, can induce the expression of  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$  and  $\beta 1$  integrins in the PNS (Ekstrom et al., 2003; Vogelezang et al., 2001; Wallquist et al., 2004; Yanagida et al., 1999). The ability of a particular ECM component to induce PTEN expression is dependent on the repertoire of integrin subunits expressed by the cell. Each  $\alpha/\beta$  heterodimer is selective for particular ECM components, though there is considerable overlap, as several  $\alpha/\beta$  pairs recognize the same components.

In neurons, integrin activation occurs rapidly, within 2 minutes of ligand binding (Lemons and Condic, 2006), but these receptors lack intrinsic enzymatic activity (Schlaepfer and Hunter, 1998). In order for integrins to become activated, they must not only bind to the appropriate ligand, but must aggregate within the plane of the membrane (Miyamoto et al., 1995) and recruit various adapter proteins and kinases to transduce



signals (Giancotti and Ruoslahti, 1999). ECM-integrin interaction recruits non-receptor tyrosine kinase, FAK (Schaller et al., 1992), whose N-terminal domain binds directly to the  $\beta$  subunit cytoplasmic domain (Dedhar and Hannigan, 1996). This interaction induces the autophosphorylation of FAK on Y397, which creates docking sites for SH2-domain containing proteins, the Src Family Kinases (SFK). This interaction in turn results in further phosphorylation events that include cytoskeletal proteins (paxillin and tensin), adapter proteins (p130cas) and kinases (FAK itself is further activated creating binding sites for Grb2-SOS complex which activates the Ras-MAPK cascade). Additionally, phosphorylated FAK can interact with PI3K, both directly and via Src, and some integrin subunits can recruit Shc, which also activates Ras-MAPK signalling (reviewed in Giancotti and Ruoslahti, 1999; Kumar, 1998; Miranti and Brugge, 2002). Furthermore, integrin-linked kinase (ILK) is a serine/threonine kinase with scaffolding functions that directly associates with the cytoplasmic tail of the  $\beta 1$  subunit (Hannigan et al., 1996), and serves as a key regulator of adhesion-dependent signal transduction (Mills et al., 2006). The genetic deletion of ILK mimics the phenotypes observed with integrin  $\beta 1$  deletion (Graus-Porta et al., 2001), and ILK is critical to cerebellar development (Mills et al., 2006), as is PTEN (Marino et al., 2002). Focal adhesion associated proteins, including FAK and Src, often contain multiple domains which can each link together a number of other proteins creating a web-like effect that is difficult to trace the sequence of activation signals from integrin ligation to signal transduction cascade (Zaidel-Bar et al., 2007). Our results show that interference with FAK, Src or ILK is sufficient to impair the LN-mediated upregulation of PTEN.

A Src-related signalling cascade induced by integrin ligation to a collagen substrate has been recently implicated in the induction of Egr-1 expression which results in the promotion of an invasive phenotype of ovarian carcinoma (Barbolina et al., 2007). Our results demonstrate that integrin activation by a LN substrate also results in the induction of Egr-1. Egr-1 is classified as an immediate early gene (Knapska and Kaczmarek, 2004), and the activation of Egr-1 synthesis by ultraviolet radiation has been shown to directly regulate expression of PTEN by binding a GC-rich site within the 5'UTR (Virolle et al., 2001), a response that was lost in Egr-1 deficient mice. My results are in agreement with those of Virolle (2001), as Egr-1 suppression using siRNA was sufficient to impair the LN-induced upregulation of PTEN. Multiple biological roles are attributable to Egr-1, including neurite outgrowth, plasticity, growth control and apoptosis (Thiel and Cibelli, 2002), and these largely overlap with roles attributable to PTEN.

Integrin receptors are by no means limited to neurons, but are in fact expressed by a wide variety of cell types. PTEN induction has previously been associated with  $\alpha 4\beta 1$  ligation in fibroblasts, where it is thought to prevent invasive phenotype associated with pulmonary fibrosis (White et al., 2003). PTEN plays a major role in anoikis, apoptosis caused by loss of attachment to the ECM (Lu et al., 1999) and the loss of PTEN is associated with a variety of human pathologies, including cancer (Salmena et al., 2008) and rheumatoid arthritis (Pap et al., 2000). Integrin receptors and their downstream signalling cascades, including PTEN induction, play an important role in embryonic development, regenerative axon repair, migratory responses and a plethora of



pathological conditions. Elucidating regulatory mechanisms may provide clues to therapeutic targets in the future.

## **Chapter 10: General Discussion and Future Directions**

The goal of the present series of studies was to examine the molecular mechanisms underlying successful axonal regeneration, specifically signalling cascades initiated by neurotrophin and ECM ligands binding to their cognate receptors, and determine how these signalling cascades might interact or cooperate to result in the promotion of an optimized growth response. The success or failure of axonal regeneration following nerve injury is largely attributable to factors present in the extracellular environment. The promotion of regenerative growth requires the orchestration of a multitude of signalling cascades which can be activated to varying degrees by a variety of different surface receptors depending on the availability of trophic and tropic cues in the extracellular environment, and the presence of inhibitory components which can activate parallel or antagonistic signalling that is counterproductive to growth. As such, the failure of CNS regenerative growth is ascribed to a lack of growth promoting factors, as well as the presence of numerous inhibitory components derived from myelin and the glial scar. Conversely, the success of axonal regeneration following peripheral nerve injury is ascribed to the presence of neurotrophic factors, including NGF, and permissive ECM components, including LN. These growth promoting molecules are often upregulated following axonal injury. The receptors for these cues also display altered expression patterns in response to injury, and are inevitably important to the cellular regenerative response.

### 10.1 p75NTR upregulation in the presence of neurotrophins

Ligation of TrkA, the high affinity NGF receptor, is well known to initiate signalling cascades for growth and survival of the developing nervous system via well characterized Ras-MAPK, PLC $\gamma$  and PI3K signalling cascades (Obermeier et al., 1993b; Stephens et al., 1994). Functions of p75NTR, the low affinity pan-neurotrophin receptor have been extensively investigated (Reviewed in Blochl and Blochl, 2007; Roux and Barker, 2002), and this receptor is recognized to participate in a multitude of functions which are often paradoxical in nature and can involve promotion of survival or apoptosis, and the promotion or inhibition of neurite growth depending upon cellular context. This pleiotropic receptor can signal in ligand-dependent and ligand-independent fashion, and the outcome is dependent upon activation status of a variety of co-receptors, including TrkA, thus the contribution of p75NTR to regenerative growth is far less clearly delineated.

It is well recognized that NGF upregulates p75NTR expression in multiple neuron types *in vivo* (Lindsay et al., 1990) and *in vitro* (Kitzman et al., 1998; Wyatt and Davies, 1993), and in PC12 cells (Doherty et al., 1988). The upregulation of p75NTR is associated with both nerve injury and NGF exposure, and induction in the developing nervous system is particularly robust in the period preceding target innervations, suggesting that the increase in p75NTR expression could be contributing to a neurite outgrowth response both during development and following injury (Goettl et al., 2004; Wyatt and Davies, 1993). Thus the mechanism of p75NTR upregulation could be critical to the regenerative response. In my initial studies, I investigated whether the individual phosphorylation sites present on the cytoplasmic tail of TrkA, and their associated



signalling cascades, contributed to the regulation of p75NTR expression in both the presence and the absence of NGF. Using a series of PC12 derivative cell lines expressing either no TrkA (PC12nnr5 cells) or TrkA mutated to eliminate Y490, Y785 or both tyrosine phosphorylation sites of the TrkA cytoplasmic tail, I demonstrated that constitutive p75NTR expression was significantly reduced in the absence of TrkA expression, and could be restored to different degrees by the introduction of TrkA mutated to abrogate Y490 or Y490/785, but not by introduction of TrkA mutated to abrogate Y785 (Figure 2.1). I further demonstrated that exposure of these PC12 derivatives to NGF resulted in increased p75NTR expression only in those cells expressing TrkA with a functional Y785 phosphorylation site. Only the parental line and the derivative expressing TrkA Y490F responded to NGF by upregulating p75NTR expression above the unstimulated condition (Figure 2.4). These results suggest that p75NTR expression is regulated by TrkA-induced activation of PLC $\gamma$ , since loss of the PLC $\gamma$  interaction site (Y785) resulted in low levels of p75NTR and failure to upregulate in response to NGF. This hypothesis was subsequently confirmed in my second study, using pharmacological inhibition of PLC $\gamma$ . PLC $\gamma$  activity results in generation of IP3 and DAG, which subsequently activates a variety of PKC isoforms. PKC $\delta$  is of particular importance to neurite outgrowth in PC12 cells (Corbit et al., 1999) and has previously been associated with the induction of p75NTR in response to hypo-osmolarity (Peterson and Bogenmann, 2003). Its importance in p75NTR induction in response to neurotrophin stimulation was confirmed using pharmacological inhibition and siRNAs (Figure 3.6). Thus, NGF stimulation acts via TrkA Y785 phosphorylation to initiate PLC $\gamma$ -PKC $\delta$  signalling resulting in the upregulation of p75NTR.



One of my goals for this project was to apply signalling for the promotion of successful PNS regeneration to CNS neurons, and determine if similar effects are elicited. Neurotrophins can also promote the extension of CNS axons, and are especially adept at encouraging growth into grafted tissue to help overcome graft-host barriers when tissue grafts are employed (Menei et al., 1998; Xu et al., 1995a). I sought to translate my findings related to NGF-induced upregulation of p75NTR into CNS neurons, specifically CGN. CGN do not express the NGF receptor, TrkA, but do express the BDNF receptor, TrkB. All Trk isoforms activate a similar sequence of signalling events (Atwal et al., 2000; Middlemas et al., 1994; Yuen and Mobley, 1999; Zirrgiebel et al., 1995) and in fact, I demonstrated that BDNF stimulation of TrkB appears to act via PLC $\gamma$ -PKC $\delta$  signalling cascade to upregulate p75NTR in CGN (Figure 3.9).

Trk and p75NTR are coexpressed throughout much of the nervous system; typically cells that express both receptors display 10 fold more p75NTR than Trk (Chao and Hempstead, 1995). While p75NTR can signal autonomously, coexpression with Trk allows these receptors to functionally interact resulting in reciprocal modulation of function. As such, p75NTR is appreciated to enhance the function of Trk on multiple levels. p75NTR collaborates with Trk to alter cellular response to neurotrophins: its presence is found to increase the rate of NGF binding and retrograde transport, enhance Trk responsiveness when ligand levels are low, and increase the specificity of ligand-receptor interactions (Bibel et al., 1999; Clary and Reichardt, 1994; Hantzopoulos et al., 1994; Verdi et al., 1994). p75NTR has also been demonstrated to enhance the phosphorylation of Trk and its downstream intermediates, including Shc (Berg et al., 1991; Epa et al., 2004), and can influence the kinetics of Trk ubiquitination and

internalization to prolong signalling (Arevalo et al., 2006; Geetha et al., 2005; Makkerh et al., 2005). Much of this collaboration has long been believed to be due to p75NTR assisting in the formation of high affinity binding sites (Hempstead et al., 1991), a concept recently challenged by structural models and biochemical data that preclude the formation of p75NTR-Trk heterodimers (Wehrman et al., 2007). p75NTR may instead bind neurotrophin, concentrate it at the cell surface and pass it to Trk in an appropriate conformation for binding (Barker, 2007). Furthermore, the binding of NGF to p75NTR is sufficient to negate the constitutive activation of Rho, thereby removing a second form of growth inhibition (Yamashita et al., 1999). Hence the upregulation of p75NTR in response to neurotrophin stimulation may be contributing to amplification of the growth response by creating a positive feedback loop to enhance Trk signalling for growth and survival in the presence of its cognate ligand (see schematic Figure 10.1). In support, studies examining p75NTR upregulation after PNS injury demonstrate that liganded p75NTR can promote survival and regeneration, whereas unliganded p75NTR induces apoptosis (Sorensen et al., 2003) and complexes with NgR to activate Rho and inhibit growth. My results are also consistent with the observed modulation of p75NTR during development of the nervous system. In the period just preceding target innervation, final target derived neurotrophin (NGF) induces p75NTR expression in sympathetic neurons to decrease their sensitivity to NT3 (Kuruvilla et al., 2004). p75NTR expression levels are central to the regulation of neuronal survival and axonal growth in response to target derived neurotrophin and subsequently regulate innervation density in the developing

**Figure 10.1 Schematic illustration of proposed positive feedback model.** The addition of NGF to PC12 cells in culture activates two structurally distinct receptors, p75NTR and TrkA. Neurotrophin binding to p75NTR disengages the constitutive activation of Rho, which promotes neurite outgrowth. Binding of TrkA by NGF results in phosphorylation of the cytoplasmic tail which initiates specific signalling cascades. The present studies demonstrate that Trk ligation and subsequent PLC $\gamma$ -PKC $\delta$  signalling upregulates p75NTR expression, and I speculate that this also promotes neurite growth by enhancing the function of TrkA.

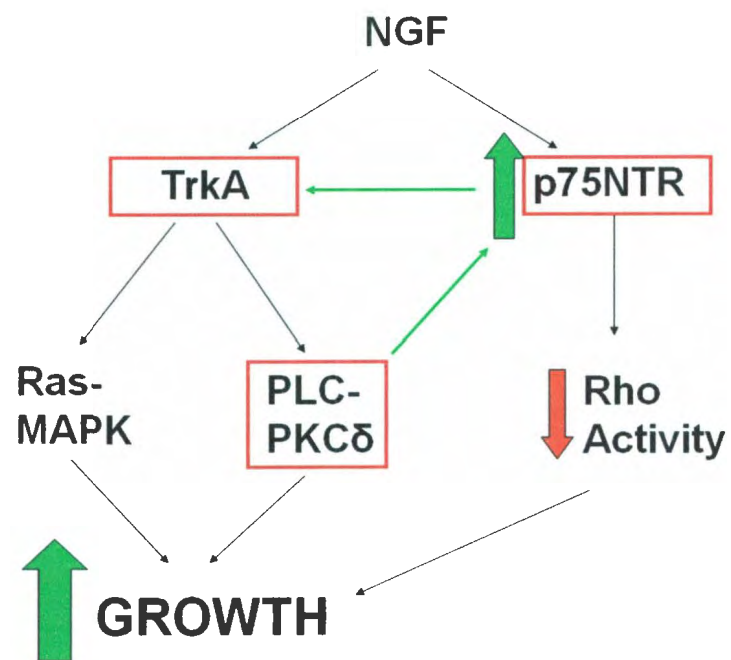


Figure 10.1



nervous system, as increased p75NTR can induce apoptosis of those neurons unsuccessful at obtaining appropriate neurotrophin support (Kuruville et al., 2004). Recently a concept of axon pruning was described wherein competing sympathetic axons secrete BDNF in an activity dependent fashion which selectively binds p75NTR to eliminate less active neurons (Singh et al., 2008). Elucidation of the mechanism of p75NTR upregulation provides insight into the regulation of essential biological processes and may yield additional insight into the process of neuronal degeneration since injury, stress and pathology are also recognized to upregulate p75NTR (Dechant and Barde, 2002; Schor, 2005).

## **10.2 Role of ECM in enhanced regenerative growth**

While my initial studies indicated that the presence of neurotrophin upregulated p75NTR expression, which I speculate would result in enhanced Trk-induced signalling for growth, it is likely that multiple factors are required to realize optimal regenerative growth. A second factor well recognized to promote regenerative axonal growth is a permissive ECM, including LN, found in abundance in the PNS (Lentz et al., 1997). ECM cues are mediated by integrin heterodimers which aggregate upon ligation exerting direct control over the actin cytoskeleton, as well as initiating biochemical signalling cascades. Integrin signalling has emerged as a key modulator of regenerative growth, and integrin ligation can promote a growth response in PNS neurons in the absence of additional neurotrophin support (Tucker et al., 2005; Tucker et al., 2006; Tucker et al., 2008). Furthermore, integrin signalling can potentiate growth promotion induced by

neurotrophin stimulation, synergistically or cooperatively activating pathways necessary for growth (Tucker et al., 2005; Tucker et al., 2006; Tucker et al., 2008).

My third study examined the contribution of the ECM component LN to regenerative growth of a sympathetic neuron model. I demonstrated that both LN and NGF are capable of independently eliciting growth from PC12 cells, but the combination of both stimuli promotes more robust response than either alone (Figure 4.1). Because ECM proteins and neurotrophins utilize similar intracellular signalling pathways to promote axon elongation, my initial investigations focused on enhanced activation of intermediates of known importance to sympathetic neurite growth, including ERK. I determined that LN was able to phosphorylate ERK autonomously, but also contributed to the enhanced phosphorylation noted in response to co-stimulation with NGF. Manipulation of ERK phosphorylation using constitutively active or dominant negative Ras constructs promoted and inhibited growth of PC12 cells, respectively (Figure 4.2).

Since my initial studies indicated that NGF potentially enhances growth by increasing p75NTR expression, I speculated that LN might have similar effects on p75NTR expression, since LN associated signalling intermediates are reported to phosphorylate PKC $\delta$  (Parekh et al., 2000; Steinberg, 2004). The consequently enhanced Trk activation could provide an explanation for enhanced phosphorylation of ERK. Interestingly, I found the opposite: both Western blotting and quantitative PCR revealed that the LN substrate was associated with significantly decreased p75NTR expression (Figure 4.3, 4.4), and in fact, the overexpression of p75NTR in the absence of ligand resulted in impaired growth on LN (Figure 4.6). This effect on neurite outgrowth may be due to the influence of p75NTR on the activation of Rho, a known modulator of



cytoskeletal dynamics which is well recognized to inhibit neurite extension when activated. p75NTR constitutively activates Rho (Yamashita et al., 1999), and p75NTR siRNA has been demonstrated to decrease active Rho resulting in the promotion of neurite outgrowth (Ahmed et al., 2005). I therefore investigated LN and p75NTR effects on Rho activity to determine if this was in fact valid in this scenario. I determined that the LN substrate was associated with a decreased level of active Rho, and the overexpression of p75NTR resulted in an increased level of active Rho (Figure 4.7), thereby elucidating a mechanism for the effects of unliganded p75NTR on modulation of the cytoskeleton required for neurite outgrowth. Thus I concluded that LN is able to enhance growth, in part by diminishing the expression of a potentially inhibitory receptor, p75NTR. This decrease in p75NTR may result in decreased Rho activity, but I also speculate it could result in decreased transduction of inhibitory signals from myelin associated proteins that bind co-receptor NgR. As p75NTR has both ligand-dependent and independent signalling, its role in the growth response is largely unclear and is often cell type and context dependent. Blocking p75NTR has been shown to promote growth in several scenarios, generally those in which myelin inhibition and activation of Rho directly inhibit growth (Ahmed et al., 2005; Gehler et al., 2004; Higuchi et al., 2003a). LN can override inhibitory actions of myelin and CSPGs on CNS neurons, likely by manipulating Rho activation, which may occur via decreased p75NTR expression.

Within this study, I further determined a mechanism for integrin-mediated regulation of p75NTR (see schematic Figure 10.2). A previous report indicated that PTEN overexpression in PC12 cells resulted in decreased p75NTR expression (Musatov et al., 2004). This study, combined with my own observations and the emerging role for

**Figure 10.2 Model of LN-induced signalling cascade.** LN binding to integrin receptors promotes neurite growth and motility. Integrin signalling through FAK, Src and ILK, and the downstream induction of transcription factor Egr-1 results in the upregulation of PTEN. PTEN translocates to the nucleus where it decreases the DNA binding ability of Sp1, resulting in decreased transcription of p75NTR. As p75NTR constitutively activates Rho, decreased p75NTR expression results in decreased Rho activity which promotes neurite outgrowth. Furthermore, LN signals result in the phosphorylation of ERK, which promotes growth in PC12 cells and sympathetic neurons. LN is also associated with enhanced MMP activity, possibly contributing to the decrease in p75NTR expression, and the decrease in p75NTR could further enhance growth by reducing the transduction of myelin associated inhibition.



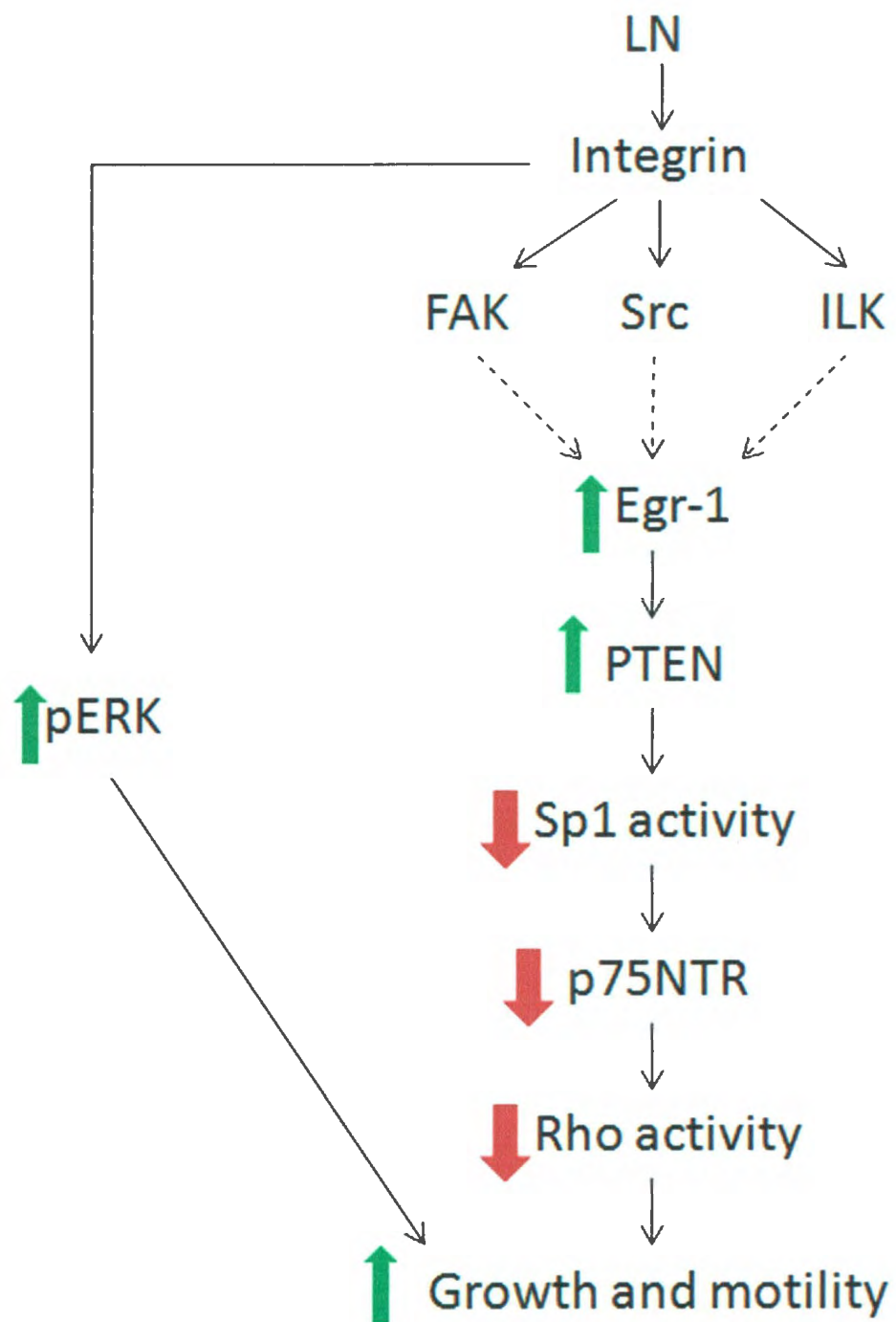


Figure 10.2

PTEN in the development of the nervous system (van Diepen and Eickholt, 2008) led me to investigate a possible role for PTEN in this signalling cascade. I determined that LN substrate upregulated PTEN expression in an integrin-dependent manner (Figure 4.9), and used PTEN siRNA and overexpression techniques to demonstrate a consistently inverse relationship between PTEN and p75NTR expression (Figure 4.11). Furthermore, PTEN siRNA impaired neurite extension in response to a LN substrate.

A more detailed investigation of the relationship between PTEN and p75NTR was performed to establish if there was a direct relationship or simply a correlation between expression levels. As PTEN has the ability to dephosphorylate both lipid and protein substrates, I used a series of mutated PTEN constructs to establish which of these phosphatase activities was responsible for the downregulation of p75NTR expression. I hypothesized that the lipid phosphatase activity could be negatively impacting the PLC $\gamma$  signalling previously established to upregulate p75NTR. However, I again demonstrated the opposite was true: while the overexpression of a phosphatase-dead PTEN (C124S) had no impact on p75NTR expression, the overexpression of either the wild-type PTEN or a mutant version that is devoid of lipid phosphatase activity but retains protein phosphatase activity (G129E) both resulted in decreased p75NTR expression levels (Figure 7.1). These results suggested the protein phosphatase activity of PTEN was responsible for modulation of p75NTR expression.

While the protein phosphatase activity of PTEN is far less studied than the lipid phosphatase activities, a few protein substrates have been demonstrated to be dephosphorylated by PTEN. Of interest to my studies was the report that PTEN could dephosphorylate FAK and Shc (Tamura et al., 1998), which was associated with impaired

migratory abilities of fibroblasts. My results, however, indicated that the LN substrate was associated with increased phosphorylation of FAK (data not shown) despite enhanced PTEN expression, and we were further unable to detect any impact of PTEN overexpression on the phosphorylation of FAK. Therefore, while the concept of dephosphorylating FAK is attractive, as focal adhesion turnover is necessary for the extension of axons and cell migration, it is not necessarily the case in this system, and may require further analysis to determine if it is occurring in a spatially localized manner and thus undetectable by Western blotting.

In an attempt to determine where PTEN was localizing, I performed immunocytochemistry, which revealed a large number of cells that appeared to have PTEN localized to the nucleus on the LN substrate, but not on the PL substrate (Figure 7.2). Nuclear PTEN has become a well-documented phenomenon (Barker, 2007), despite the absence of a canonical nuclear localization sequence. It is instead reported that PTEN enters the nucleus either via passive diffusion, or through calcium dependent association with a major vault protein that acts as a carrier (Chung et al., 2005; Liu et al., 2005; Minaguchi et al., 2006). Nuclear PTEN is notably increased during neuronal differentiation (Lachyankar et al., 2000) and several functions for the nuclear pool of PTEN have been proposed: as nuclear PIP<sub>3</sub> appears to be insensitive to PTEN (Lindsay et al., 2006), alternate demonstrated functions include maintenance of chromosomal integrity (Shen et al., 2007), protein-protein interactions to stabilize p53 (Li et al., 2006; Lian and Di Cristofano, 2005) and a clear influence over transcriptional activities. In this regard, transcription factor, Sp1, represents another protein substrate demonstrated to be dephosphorylated by PTEN (Kang-Park et al., 2003). Since Sp1 is reported to upregulate



p75NTR expression under conditions of hypo-osmolarity (Ramos et al., 2007), and LN is reported to decrease Sp1 activity (Gaudreault et al., 2007), the effect of PTEN on Sp1 phosphorylation was potentially of key importance to this mechanism. As the phosphorylation status of Sp1 is a critical regulator of its DNA binding ability (Bouwman and Philipsen, 2002; Li et al., 2004), I used EMSA and ChIP assays to demonstrate that both the LN substrate and PTEN overexpression decreased the ability of Sp1 to bind to the p75NTR promoter (Figure 7.4), which results in decreased transcription (Figure 4.4) and the ultimate downregulation of p75NTR protein expression. Thus it appears that LN-induced PTEN can decrease the ability of Sp1 to transcribe p75NTR, and I speculate that this is occurring by dephosphorylation of Sp1, though I have not shown this. Of note to my earlier studies, PKC has been demonstrated to phosphorylate Sp1 (Kang-Park et al., 2003), thereby increasing its DNA binding ability, and thus providing a potential mechanism for the upregulation of p75NTR in response to neurotrophin stimulation.

These studies established both a novel role for PTEN in the regulation of p75NTR expression and neurite outgrowth, and a novel mechanism for the regulation of PTEN, an important tumour suppressor with functions well beyond the nervous system. Questions remain as to the physiological signals which regulate PTEN localization, as well as stability and activity and these areas require additional investigation. Furthermore, Sp1 is a highly prevalent transcription factor which controls the expression of several genes. There is the distinct possibility that PTEN regulation of Sp1 DNA binding ability has broader downstream implications that also warrant further investigation.

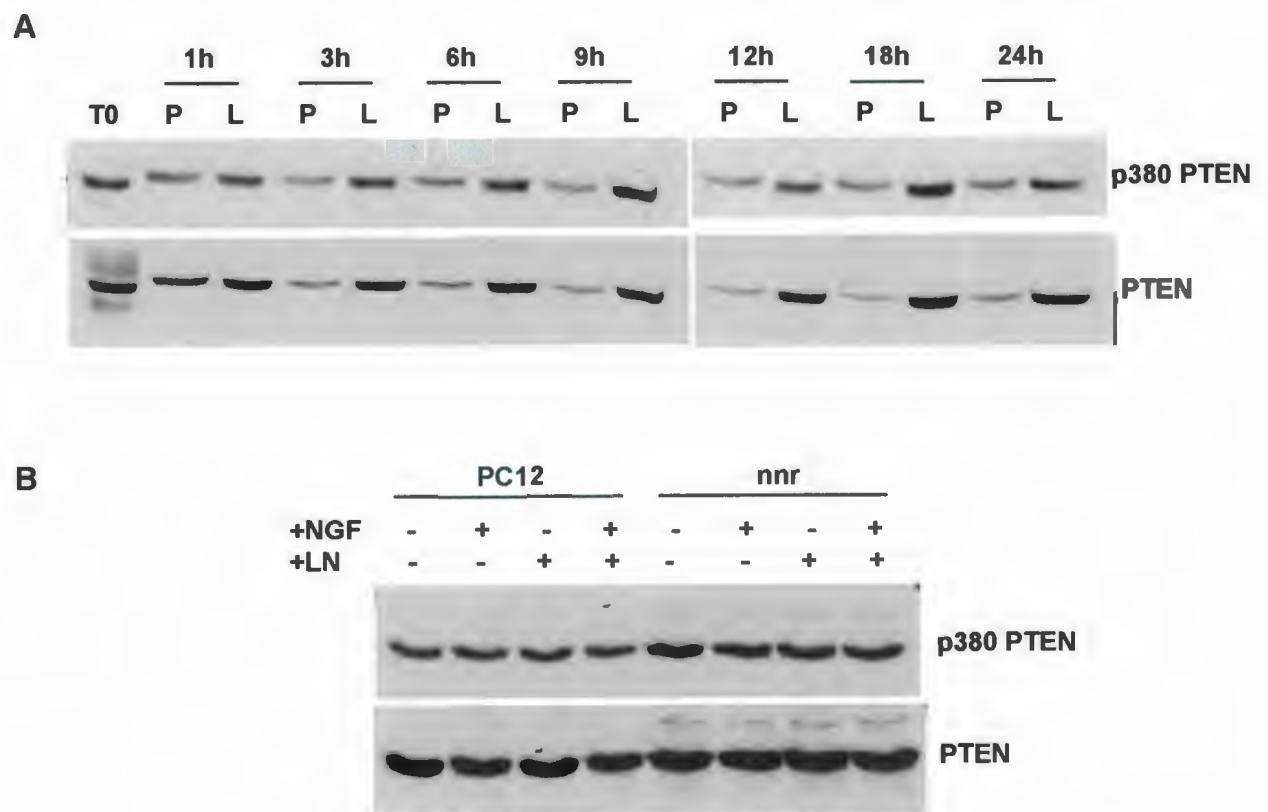
Both in its role as a tumour suppressor and a PI3K antagonist, PTEN is associated with impaired survival (Li et al., 1998a; Toker and Newton, 2000). The lack of apoptosis



observed on a LN substrate can partially be explained by the compensatory upregulation of total Akt, but may also be reconciled by examination of PTEN phosphorylation. In order for PTEN to act as a lipid phosphatase and antagonize PI3K to impair the phosphorylation of Akt and downstream survival signals, it must be present in an active form. Phosphorylation of S380, T382 and T383 residues of the C-terminus renders PTEN catalytically inactive against lipid substrates (Odriezola et al., 2007), and thus unable to negatively impact the phosphorylation of Akt. In contrast to PTEN induction by noxious stimuli, PTEN induced by integrin activation is largely phosphorylated as detected by an antibody against phosphorylation of S380 on a Western blot (see preliminary data in Figure 10.3). While the ratio of phosphorylated PTEN to total PTEN is not significantly different between the PL and LN conditions, the fact that the induced PTEN is present in a phosphorylated state ensures that there are no negative survival-related consequences associated with the upregulation of PTEN. The impact of phosphorylation on protein phosphatase activity of PTEN remains to be determined, but our data would suggest that it remains functional. In addition to inhibiting PTEN activity directly, the phosphorylation of the C-terminus is also required for stability of the protein and may impact its ability to associate with other proteins or localize (Tolkacheva et al., 2001; Vazquez et al., 2001; Vazquez et al., 2000). The phosphorylation of particular sites of the C-terminus is due to the actions of kinases CK2 and PICT-1 (Okahara et al., 2006; Torres and Pulido, 2001; Yim et al., 2007). Further investigation into the impact of integrin activation on these kinases may yield additional insight into the regulation of PTEN activity, expression level and localization, and whether the outcome involves apoptosis or growth. As well, stimuli necessary for autodephosphorylation of PTEN and the

**Figure 10.3 PTEN induced by integrin activation is phosphorylated on Ser 380. A)**

Cells plated on PL vs LN for the indicated period of time were harvested for protein analysis. Results indicate that the LN-induced PTEN is highly phosphorylated on S380 as early as 1h post LN exposure. B) A comparison of PC12 vs nnr cells subjected to short-term (10 min) stimulation with NGF, LN, or both, reveals no influence of these stimuli on the phosphorylation of PTEN, but does reveal that the nnr cells, which express constitutively higher PTEN, also have constitutively higher level of phosphorylated PTEN.



**Figure 10.3**

identification of other phosphatases that could regulate PTEN phosphorylation status remain undetermined.

Again, one of the main objectives of this project was to elucidate signalling mechanisms induced by the extracellular environment to promote successful axonal regeneration in PNS neurons and translate these findings into CNS neurons to determine if similar signalling mechanisms can be exploited to promote regenerative growth in the CNS. For this reason, I analyzed the presence of this LN-mediated cascade in primary neonatal hippocampal neurons and determined that LN was able to induce similar changes in PTEN and p75NTR, which were again associated with enhanced neurite outgrowth (Figure 4.14). This may indicate a role for LN-induced signals in hippocampal development. Whether or not this cascade could be utilized to promote regenerative growth after injury in the CNS warrants further investigation. I have also analyzed the presence of this LN-mediated cascade in primary CGN, where I determined that while a similar signalling cascade exists (Figure 6.1), it persisted beyond the establishment of axonal outgrowth, but notably ceased in correlation with the temporal period of developmental migration (Figure 6.3), suggesting perhaps another function for this LN-induced signalling cascade.

### **10.3 Regulation of PTEN-p75NTR signalling for migration**

There is vast overlap between the processes of cytoskeletal remodelling that apply to both growth and motility. In fact, integrin signalling is key to cell migration, and the manipulation of Rho activity is well recognized to influence both neurite growth and



motility (Moissoglu and Schwartz, 2006; Ridley et al., 2003). Furthermore, previous studies have determined that PTEN and p75NTR have roles in proper lamina formation and foliation of the cerebellum respectively (Backman et al., 2001; Groszer et al., 2001; Kwon et al., 2001; Marino et al., 2002; Nayeem et al., 2007), which are both processes dependent upon appropriate migration. For these reasons, I developed a novel motility assay that would allow the quantitative study of population movement across an applied LN substrate (Figure 5.1), and subsequently utilized this assay to investigate the contributions of PTEN, p75NTR and Rho to LN-induced migration of CGN. I demonstrated that manipulation of any one of these pathway components can influence motility: PTEN siRNA and p75NTR overexpression each inhibit cell migration (Figures 6.5, 6.6), whereas Rock inhibition can both promote migration and rescue the migratory defects imposed by the aforementioned treatments (Figures 6.7, 6.8). LN is well recognized for its role promoting growth and migration in the developing cerebellum. As such, this signalling cascade could be crucial to the development of appropriate cerebellar architecture.

The influence of PTEN on cell migration appears to be cell type specific. PTEN has been shown to suppress migration of a variety of cell types, as genetic ablation of PTEN enhances the migration of mouse embryonic fibroblasts and PTEN reconstitution or overexpression can inhibit the migration of fibroblasts and various tumour cells (Liliental et al., 2000; Lim et al., 2004; Suzuki et al., 2003). Interestingly, the ability of PTEN to impair migration is often linked to its protein phosphatase activity (Leslie et al., 2007) and proposed mechanisms include effects on small G proteins (Liliental et al., 2000) and the direct dephosphorylation of FAK (Tamura et al., 1998). However, PTEN

deletion has no impact on levels of phosphorylated FAK and an independent study could not demonstrate PTEN-FAK interaction or FAK dephosphorylation upon PTEN overexpression (Maier et al., 1999; Sun et al., 1999). Nonetheless, selective genetic deletion of PTEN in the cerebellum results in animals that display CGN ectopia, distorted foliation and architectural abnormalities typically attributed to poor migration (Backman et al., 2001; Groszer et al., 2001; Kwon et al., 2001; Marino et al., 2002; Nayeem et al., 2007). This suggests that PTEN is critical for the promotion of appropriate CGN migration during development.

#### **10.4 Regulation of PTEN expression**

Since PTEN appears critical for the regulation of cytoskeletal remodelling for growth and migration, my final studies examined the modulation of PTEN expression. Having established previously that Trk modulates p75NTR expression in both the presence and absence of ligand, and that PTEN is of key importance to ECM-mediated regulation of p75NTR expression, I sought to determine if these two seemingly independent signalling cascades intersected upstream of p75NTR transcription. I previously demonstrated that LN-induced PTEN upregulation had no effect on TrkA expression, and thus speculated that TrkA may modulate the expression level of PTEN. I was able to show that TrkA reconstitution in Trk-deficient cells (PC12nnr5) resulted in decreased PTEN expression (Figure 8.1), and that TrkA inhibition using K252a or antisense oligonucleotides resulted in increased PTEN expression in a temporally appropriate timecourse for downstream regulation of p75NTR. Furthermore, the TrkA



mutations eliminating phosphorylation sites on the cytoplasmic tail also altered PTEN expression (Figure 8.2) in a manner inverse to that previously noted for p75NTR (Figure 2.1). Specifically, loss of TrkA or just the Y785 phosphorylation site resulted in significantly elevated PTEN levels. Conversely, loss of the Y490 phosphorylation site resulted in no detectable PTEN protein expression. Y490F results in the inability to activate the Ras-MAPK signalling cascade. Previous studies have linked activity of the Ras-MAPK cascade with aberrant downregulation of PTEN in epithelial and fibroblast cells types, as well as in particular cancers (Chow et al., 2007; Vasudevan et al., 2007); all systems in which PTEN is able to prevent cell migration and invasiveness. It would appear the neuronal response is again opposite to that of cancer, as loss of the Ras-MAPK pathway is associated with significant downregulation of PTEN and loss of PTEN is associated with impaired ability to migrate across a LN substrate.

I further demonstrated that this lack of detectable PTEN expression associated with the Y490F mutation was associated with an inability to modulate p75NTR (Figure 8.3) or Rho activity (Figure 8.4) in response to a LN substrate, and a resultant poor growth response (Figure 8.5). In contrast, the Y785F mutation resulted in very high PTEN expression and a robust response to LN in terms of both growth and motility.

TrkA modulation of PTEN in the absence of ligand may be due to constitutive low level activation of the receptor and its associated pathways, a common phenomenon when TrkA is overexpressed (Leoni and Valtorta, 2002). I demonstrated previously that the addition of NGF to cultures had no impact on PTEN expression at 24h. However, Trk activation is reported by others to have longer term effects on PTEN expression (6-8 days) (Lachyankar et al., 2000) consistent with the idea that low level activation is

affecting a response which gradually amplifies over time. Either way, it is clear that Trk exerts a modulatory influence over PTEN expression which may ultimately dictate constitutive levels of p75NTR expression. Unravelling the signalling cascades responsible for the Trk-induced modulation of PTEN requires further investigation (see schematic Figure 10.4).

I have further shown that PTEN expression is also regulated by the ECM. As the ECM clearly regulates PTEN expression resulting in downstream influence over cytoskeletal remodelling for neurite outgrowth, the ultimate goal was to characterize all the signal transduction steps arising from receptor-ligand interaction that culminate in a direct effect on the cytoskeleton. Towards this end, I investigated the mechanism by which LN induced the upregulation of PTEN by downstream activation of integrin associated signalling intermediates. I began by establishing that low concentrations of LN (1  $\mu\text{g/mL}$ ) could induce maximal induction of PTEN (Figure 9.1) and demonstrated that increased PTEN protein is evident by 1 h post-stimulation likely due to rapidly increased transcription (Figure 9.2). The ability of LN to upregulate PTEN is not specific to neurons or neuronal cell lines, nor is it specific to LN. I demonstrate that exposure to a variety of integrin-activating substrates upregulated PTEN expression and subsequently downregulated p75NTR expression. Cells appear able to respond to any substrate for which they express the appropriate integrin heterodimers to recognize, as each of these substrates, LN, MG, Col, FN, were able to induce PTEN upregulation (Figure 9.4). This did not however extend to all substrates. Exposure of PC12 cells to inhibitory substrates including CSPG-A and CSPG-C did not induce PTEN upregulation and did not impact p75NTR expression (see preliminary data Figure 10.5). It would be interesting to see



**Figure 10.4 Schematic illustration of role of TrkA in the regulation of PTEN and p75NTR expression.** TrkA overexpression results in the downregulation of PTEN, possibly due in part to the constitutive activation of the receptor frequently noted in cases of overexpression. As loss of the Y785 phosphorylation site is associated with an increase in PTEN, this suggests a role for PLC $\gamma$  signalling in the suppression of PTEN. Earlier results demonstrate that PLC $\gamma$ -PKC $\delta$  is associated with an upregulation of p75NTR in response to neurotrophin stimulation.

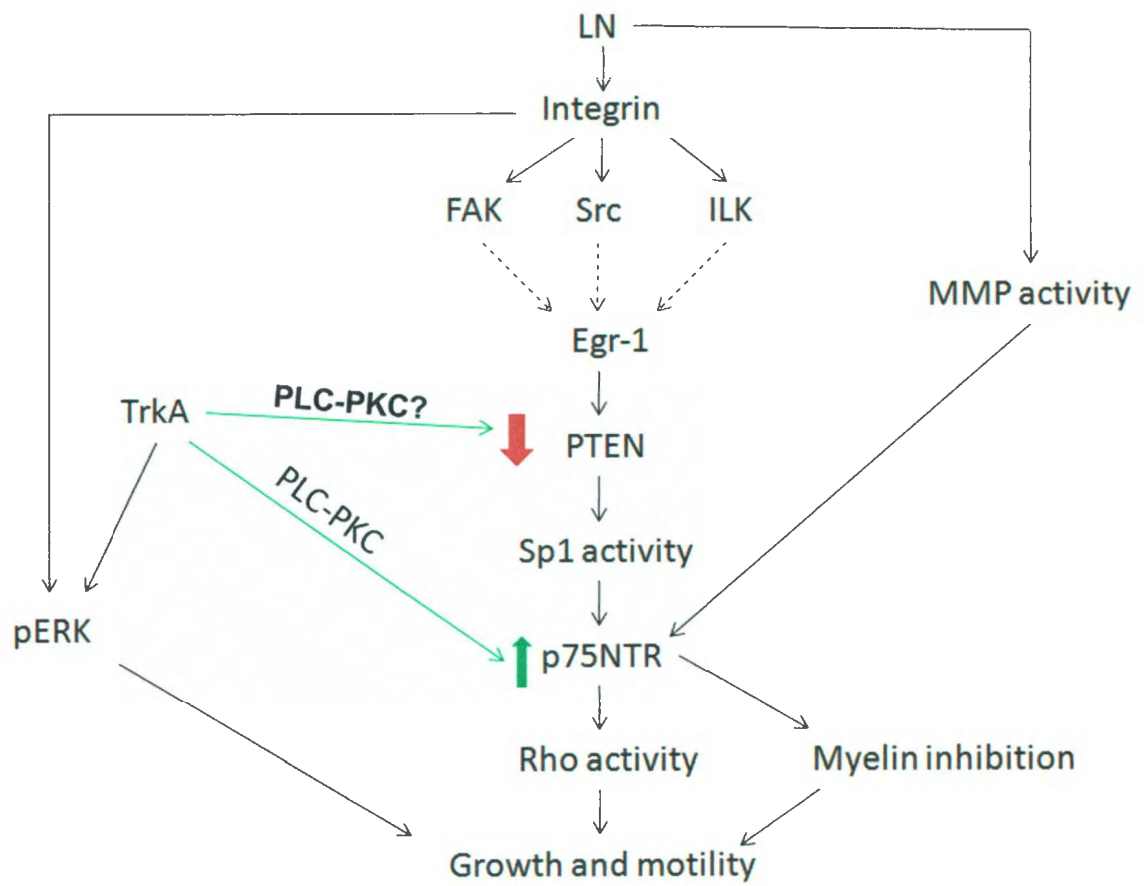


Figure 10.4

**Figure 10.5 A chondroitin sulfate substrate does not induce PTEN expression.** PC12 cells cultured on CSPG-A or CSPG-C for 24 h did not upregulate PTEN expression (A) or downregulate p75NTR expression (B) relative to a PL control substrate. Values represent the mean of 3 independent experiments  $\pm$  SEM, relative to actin.

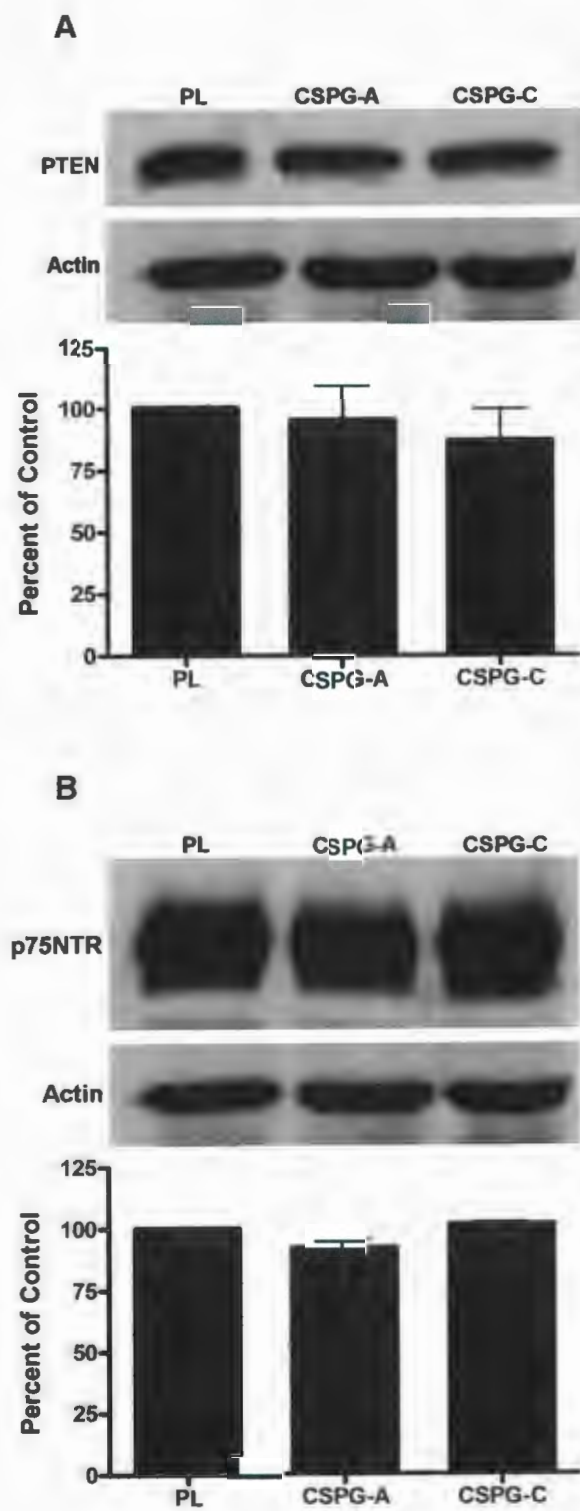


Figure 10.5



how PC12 cells respond to myelin substrates in terms of PTEN-p75NTR regulation, as well as the impact of myelin on the phosphorylation status of PTEN C-terminus, as phosphorylation is crucial to regulating PTEN stability and catalytic activity.

Since integrin ligation results in the formation of focal adhesions and the activation of biochemical signalling cascades regardless of ligand:heterodimer interaction, this suggests that focal adhesion components may be involved in signalling for the induction of PTEN. I show that this is indeed the case, as inhibition of Src using PP2 inhibited the induction of PTEN on LN in a dose-dependent manner (Figure 9.5). Furthermore, inhibition of FAK or ILK using dominant negative constructs yields a more modest impairment of PTEN induction (Figures 9.6, 9.7), but this may be due to interactions within the focal adhesion that promote or inhibit the activity of other focal adhesion components, or it may be a direct impact on the PTEN induction cascade; this remains to be determined.

LN is associated with the induction of transcription factor, Egr-1 in several model systems, including vascular smooth muscle cells and synoviotes (Morawietz et al., 1999; Warstat et al., 2008). This appears to be the case in PC12 cells as well, as LN induces increased Egr-1 expression in wild type and Y785F cell lines, but not in the Y490F line (Figure 9.8). Egr-1 is reported to activate rapidly upon stimulation and induces PTEN expression in response to UV irradiation, a response lost in Egr-1 knockout mice (Virolle et al., 2001). The induction of Egr-1 in our model system correlates with PTEN expression levels, and Egr-1 siRNA was sufficient to prevent the induction of PTEN in response to LN exposure (Figure 9.8). These results suggest that LN signalling through

integrin focal adhesion components, Src, FAK and ILK results in the rapid activation of Egr-1 which in turn induces PTEN upregulation (see schematic Figure 10.1).

The regulation of PTEN expression is vastly understudied. PTEN is a non-redundant protein whose loss is associated with a variety of human cancers, and its regulation is therefore very important and likely tightly controlled. The exact pattern of regulation throughout development remains unclear, but it is reported that PTEN is transcriptionally upregulated not only by Egr-1 but also by PPAR $\gamma$ , p53 and Sprouty2, and is downregulated by TGF- $\beta$  (Edwin et al., 2006; Patel et al., 2001; Stambolic et al., 2001). It would be interesting to determine if any of these other transcription factors have a role in LN-mediated induction of PTEN, or if it is solely attributable to the actions of Egr-1. Further regulation of PTEN activity is accomplished by phosphorylation, acetylation, oxidation and controlled localization (Tamguney and Stokoe, 2007). Stability of PTEN protein is also modulated by phosphorylation, but is further subject to protein-protein interactions. Notably an interaction between PTEN and MAGI-2 is facilitated by focal adhesion component, vinculin, and this is necessary for the prevention of PTEN degradation, as vinculin knockout mice display no PTEN protein despite normal RNA levels (Subauste et al., 2005).

The roles of PTEN in development, normal physiology as well as pathological states are just beginning to be unravelled, but an important novel role for PTEN outlined in these studies appears to be the regulation of p75NTR expression, which in turn is key to the modulation of cytoskeletal remodelling to promote growth and migration. By downregulating p75NTR in the absence of neurotrophins, LN-induced PTEN is acting to decrease the presence of a potentially inhibitory receptor, thereby tipping the balance in



favour of regenerative growth. Regeneration is more likely to succeed when local permissive signals balance and exceed inhibitory signals from the extracellular environment.

Taken together in the context of current literature, my studies collectively suggest that p75NTR is emerging as a flexible receptor that can be manipulated, not just evolutionarily (Blochl and Blochl, 2007), but acutely by contextually available external cues to achieve cellular goals of growth and motility in both PNS and CNS neurons. p75NTR expression is under the control of multiple signalling pathways, the balance of which decides the ultimate expression and consequent downstream effects. Both the addition of NGF and the decrease in p75NTR expression exerted by LN ultimately lead to the disengagement of Rho activity which promotes cytoskeletal rearrangements necessary for growth and migration. The regulation of p75NTR expression levels impacts a variety of developmental processes, including innervations patterning and axonal pruning, growth and guidance of both developing and regenerating axons. The dysregulation of both PTEN and p75NTR expression are key to pathology of various disorders and determination of regulatory cascades could lead to the identification of therapeutic targets.

## 10.5 Future Directions

The results of the current studies have further supported the potential importance of the extracellular environment on the regulation of axonal regeneration. These findings raise the following issues and, among others, impose several intriguing questions:

1. I have demonstrated that LN-mediated regulation of PTEN and p75NTR can modulate cytoskeletal dynamics to enhance axonal regeneration or motility in a sympathetic neuron model, as well as in populations of CNS neurons, including neonatal hippocampal neurons and postnatal CGN. However, the outcome of signalling cascades is often cell type specific, and it remains unclear if this response applies to all neurons.

Adult sensory neurons of the DRG are also recognized to respond to LN and NGF co-stimulation with enhanced neurite outgrowth resulting from the cooperative signalling of Trk and integrin receptors (Tucker et al., 2005; Tucker et al., 2006; Tucker et al., 2008) and although the role of p75NTR in mechanisms for this enhanced growth remain largely undefined, it is reported that direct and selective activation of p75NTR impairs regenerative growth (Kimpinski et al., 1999). Furthermore, successfully regenerating DRG axons have been reported by one group to be devoid of p75NTR expression (Song et al., 2008), and p75NTR siRNA in DRG is reported to decrease the activity of Rho and promote outgrowth in the presence of myelin (Ahmed et al., 2005). Conversely, p75NTR knockout mice displayed no growth advantage of sensory neurons, but sympathetic neurons were endowed with the ability to extend over myelin in response to neurotrophins (Hannila and Kawaja, 1999).



Preliminary evidence suggests that LN modulation of PTEN and p75NTR inverse relationship and cascade is functional based on quantitative PCR analysis of mRNA transcription in the total DRG population (Figure 10.6). However, the DRG is comprised of multiple subpopulations of sensory neurons which display different growth outcomes in response to LN and NGF exposure (Tucker et al., 2005; Tucker et al., 2006). Immunocytochemical analysis reveals different PTEN and p75NTR expression levels across populations (Figure 10.7). While the inverse relationship holds true, the populations expressing high PTEN levels do not extend neurites on LN or in response to NGF (as they are likely to be GDNF responsive based on IB4+ staining). Cryosections and qPCR confirm that those neurons expressing the high levels of PTEN are in fact the IB4+ population (Figure 10.8). Both subpopulations of DRG are dependent upon PI3K-Akt signalling for growth (Tucker et al., 2005; Tucker et al., 2006). PTEN, by virtue of its antagonism of PI3K, negatively regulates the Akt cascades and thus impairs growth.

I have identified different PTEN expression levels between the NGF-responsive and NGF-nonresponsive populations of DRG, which suggests an area in which these populations differ dramatically. Exploration of this area may yield further insight into the signalling that impairs the growth potential of the IB4+ population in response to LN. In this regard, PI3K pathway focussed microarray should yield novel insights into the intrinsic differences between the populations.

Importantly, these preliminary results suggest that LN-induced PTEN-p75NTR alterations do not promote growth in all neurons for reasons that remain unclear. The role of PTEN in the DRG remains unresolved, as does its activation status. If PTEN is phosphorylated, presumably it is catalytically inactive against lipid substrates of the

**Figure 10.6 Evaluation of total DRG mRNA reveals an increase in PTEN and decrease in p75NTR in response to a LN substrate.** DRG neurons isolated and cultured on PL versus LN coated substrates for 48 h were analyzed using real time RT-PCR to quantitate mRNA levels. Results demonstrate the LN substrate is associated with an increase in PTEN and a decrease in p75NTR, consistent with results obtained for PC12 cells and CNS neurons. Values represent the mean expression of 3 independent experiments  $\pm$  SEM, relative to 28S. \* $p < 0.001$  (ANOVA).

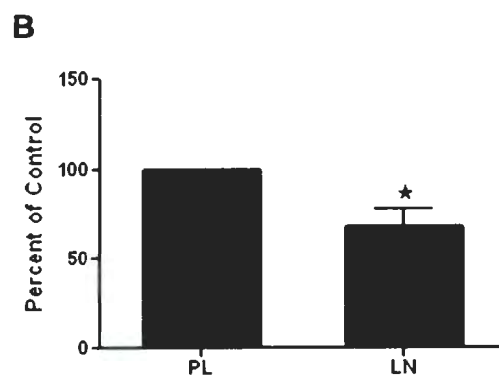
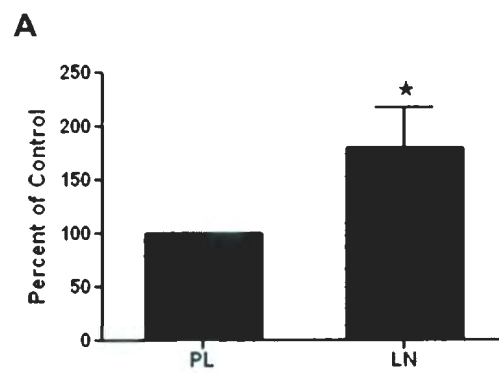


Figure 10.6

**Figure 10.7 Immunocytochemistry analysis reveals differential PTEN and p75NTR expression across DRG populations.** DRG neurons were isolated and cultured on PL vs LN coated 16 well chamber slides in the presence or absence of NGF for 24h, prior to immunostaining for PTEN (red) and p75NTR (green). Results demonstrate a clear inverse relationship between PTEN and p75NTR, as cells that are highly expressing PTEN are low in p75NTR and vice versa. Cells expressing high PTEN do not respond to either NGF or LN with neurite growth, leading us to speculate that this represents the GDNF-responsive IB4<sup>+</sup> population.



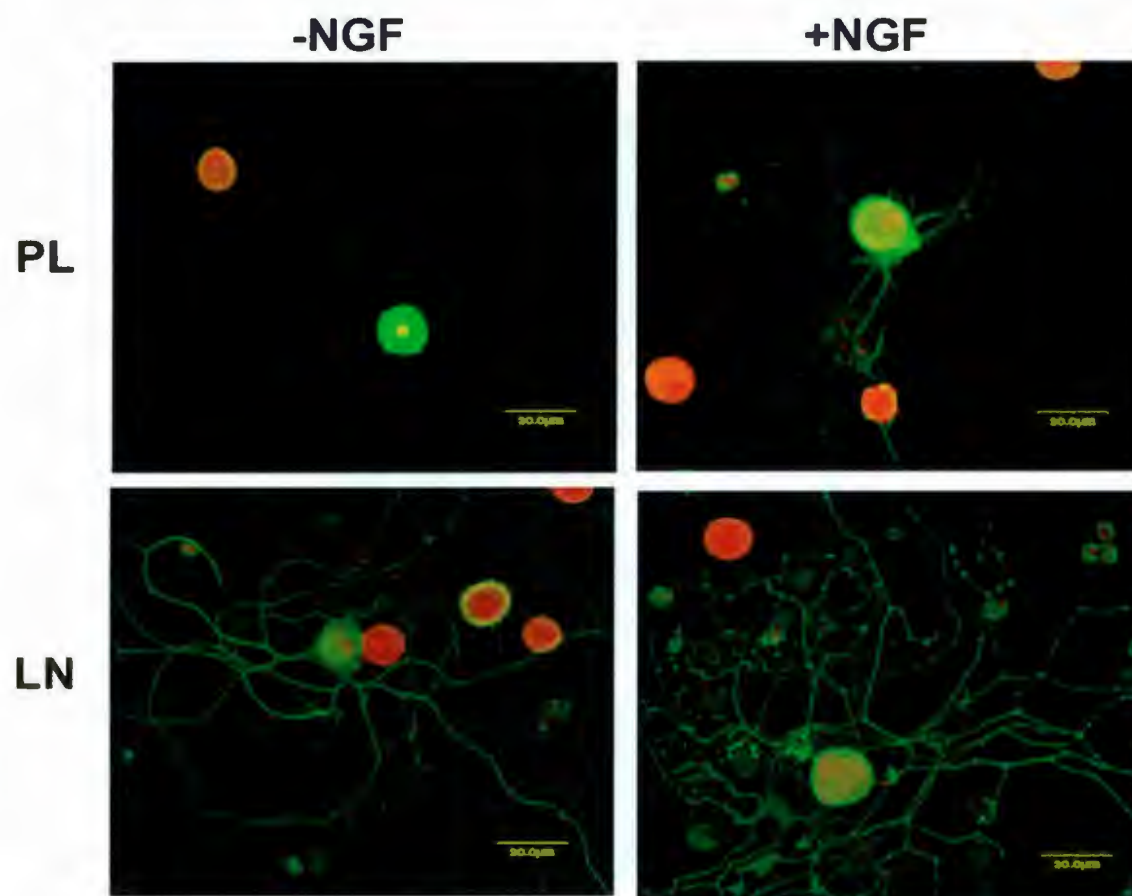


Figure 10.7

**Figure 10.8 IB4+ DRG neurons express high levels of PTEN.** Whole DRG were cryosectioned and immunostained in an attempt to determine the biochemical identity of those cells expressing high PTEN. A-C) PTEN (red) and p75NTR (green) clearly stain individual populations of DRG neurons. D-F) p75NTR and CGRP costaining reveals significant overlap between expression of patterns, as does costaining with RT-97 and p75NTR (G-I). However, RT-97 and PTEN did not appear to costain (J-L). M) IB4+ DRG neurons were selected using antibody-coated Dyna beads and separated from the NGF-responsive population (Tucker et al. 2006) Real time RT-PCR analyses of these two individual populations demonstrates that the IB4+ DRG neurons are high in PTEN expression relative to the NGF responsive population.

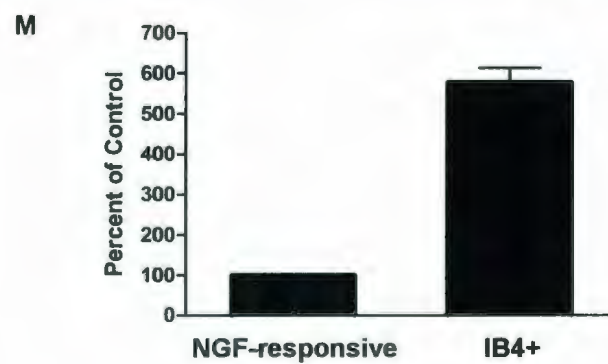
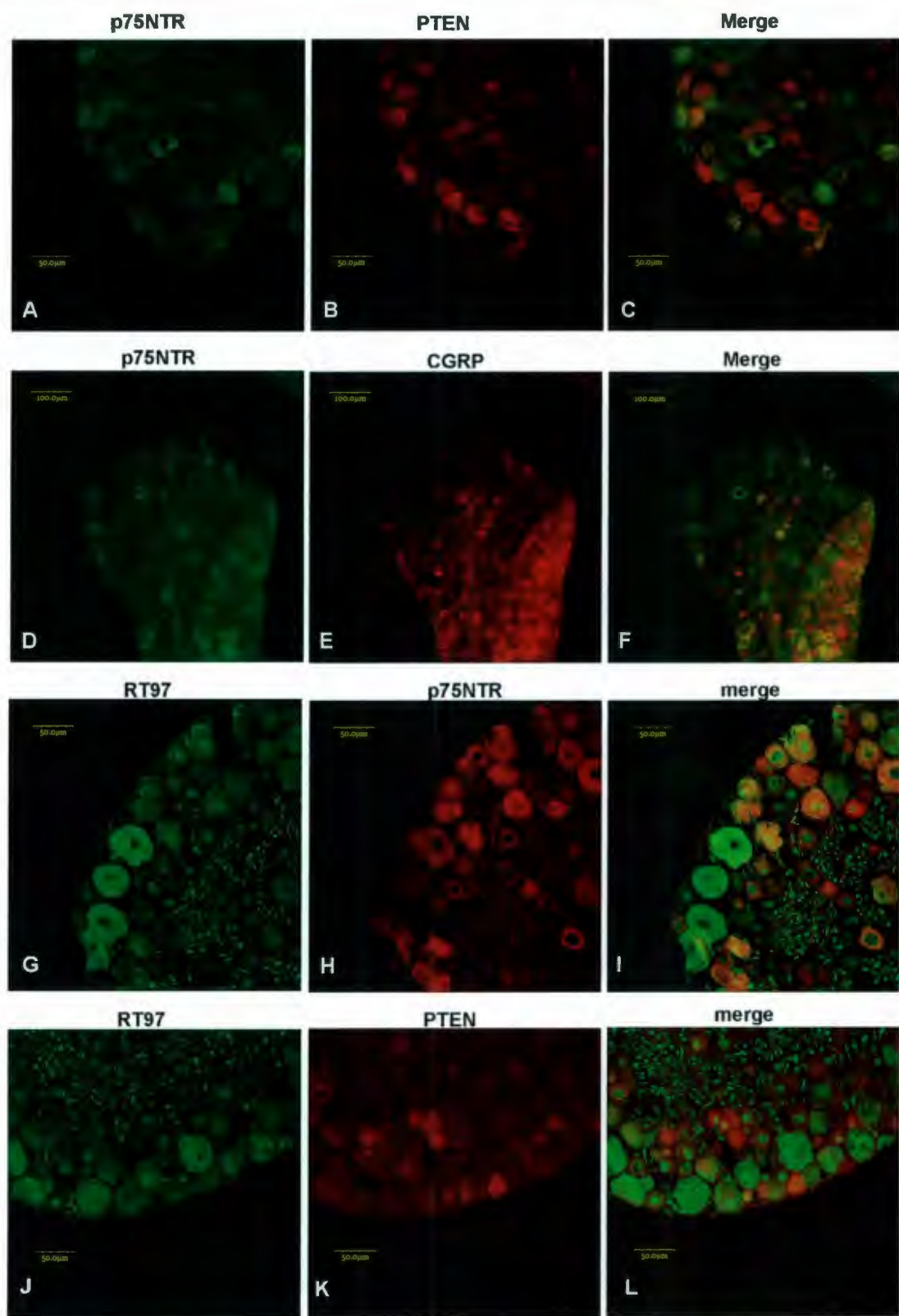


Figure 10.8

PI3K-Akt cascade. If it is not phosphorylated, this suggests differential regulation of PTEN phosphorylation between the DRG and PC12 cells, which could be due to differential kinase or phosphatase activity. Activation status of PTEN perhaps represents an area that could be subject to intervention.

2. I have thus far provided LN exposure to PC12 cells and assorted neurons in the form of a two-dimensional (2D) substrate. It has been suggested that the culture of neurons on a 2D tissue culture surface is somewhat artificial, and that the provision of a three-dimensional (3D) culture substrate would provide the neurons with a more physiologically relevant growth scenario, as the ability of cells to interact with the ECM depends on the physical and biochemical characteristics of the particular matrix, as well as cellular properties and a 3D substrate may alter contact-dependent signalling elicited by the ECM.

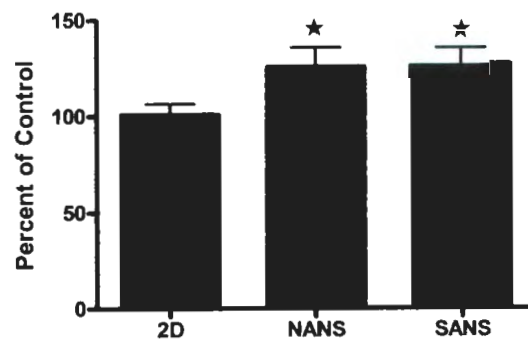
We have started to use synthetic 3D matrices to study signalling. The ultra-web synthetic nanofibrillar surfaces used in these experiments were provided by Donaldson Company Inc (Minneapolis MN, USA). CGN were plated on either 2D PL-coated tissue culture plates, or on uncoated (NANS) or polyamine coated (SANS) 3D nanofibre tissue culture dishes and subsequently examined for signalling differences to yield insight into environmental conditions required for neurite outgrowth *in vivo*. Initial investigations revealed an upregulation of PTEN and a downregulation of p75NTR associated with both of the 3D substrates relative to the 2D substrate (Figure 10.9). Subsequent MTT survival assays demonstrated enhanced survival associated with the 3D matrices relative to the 2D substrate (Figure 10.10). The enhanced PTEN expression is therefore not associated with



**Figure 10.9 A 3D substrate upregulates PTEN and downregulates p75NTR mRNA.**

CGN cultured on 2D tissue culture surface verses 3D NANS or SANS tissue culture surfaces for 8DIV were sampled and subjected to real-time RT-PCR to quantify the relative mRNA. Results demonstrate that the 3D NANS and SANS substrates were both associated with increased PTEN mRNA (A) and decreased p75NTR mRNA (B). Values expressed represent the mean of only 2 replicates +/-SEM relative to 28S. \* $p < 0.05$  (ANOVA).

**A**



**B**

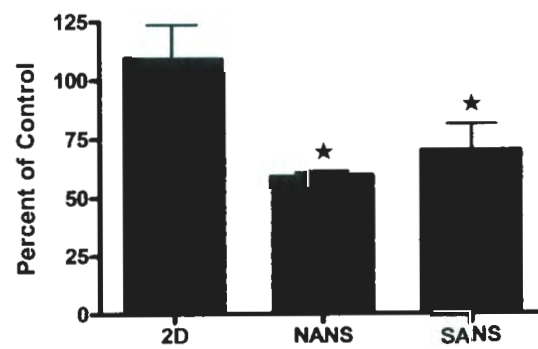
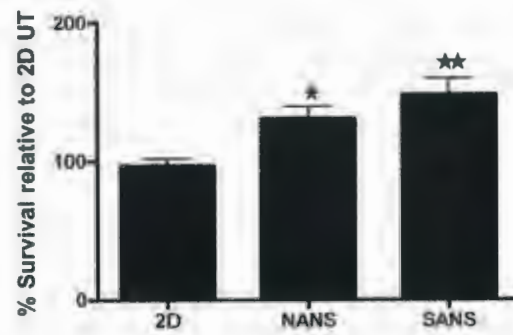


Figure 10.9

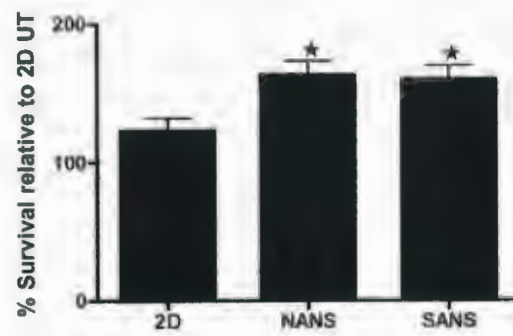
**Figure 10.10 A 3D substrate is invariably associated with enhanced neuronal**

**survival.** CGN were cultured on 2D tissue culture surfaces or 3D nanofibre substrates, NANS (uncoated) or SANS (polyamine coated) 96 well plates for 8DIV. MTT survival assay demonstrates enhanced survival of CGN on 3D substrates relative to 2D substrates when substrates are untreated (A), PL-coated (B) or LN-coated (C). Values represent the mean survival of 3 independent experiments  $\pm$  SEM expressed relative to the control condition (2D). \* $p < 0.05$ , \*\* $p < 0.01$  (ANOVA).

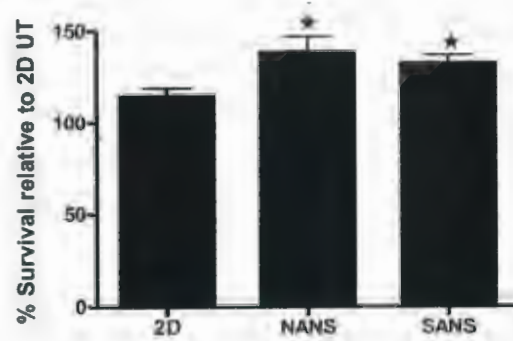
**A untreated**



**B PL coated**



**C LN coated**



**Figure 10.10**



an apoptotic response to the nanofibres, but may instead be associated with enhanced growth or motility, both of which warrant further investigation. I have also performed ECM pathway focussed microarrays to sample a wider variety of ECM associated signalling intermediates, and the preliminary results are displayed (Figure 10.11). Multiple genes are altered in response to the 2D and 3D substrates, including integrin  $\alpha 5$ , MMP9, and Lamb2 to name a few.

3. In addition to extracellular factors investigated in these studies, axonal regeneration is dependent upon the intrinsic state of the neuron, which is largely dependent upon cAMP levels. LN is able to decrease cAMP levels depending upon concentration, but notably high intracellular cAMP and laminin-mediated integrin activation together result in Rho GTPase dependent growth cone collapse (Lemons and Condic, 2006). cAMP is nonetheless recognized to promote growth over inhibitory substrates (Bandtlow, 2003; Cai et al., 2002) suggesting that cAMP acts to either alter the reception or interpretation of inhibitory cues. I therefore sought to investigate if cAMP could influence the expression of PTEN and p75NTR. Activation of cAMP in PC12 cells using Forskolin (10  $\mu$ M) decreased the expression of PTEN (Figure 10.12 A) and increased the expression of p75NTR on a LN substrate (Figure 10.12 B). This would seem to suggest that decreased PTEN and increased p75NTR elicited by forskolin could be involved in the growth cone collapse via downstream regulation of Rho, but obviously this requires further investigation.

**Figure 10.11 ECM array gene expression and representative clustergram from CGN cultured on 2D versus 3D substrates.** Representative microarray gene expression and analysis for CGN before (time 0) and after being plated on 2D PL, 3D NANS or 3D SANS substrates for 8DIV. A, A2: Clustergram and microarray of CGN immediately following isolation. B, B2: Clustergram and microarray of CGN plated on 2D PL-coated surface for 8DIV. C, C2: Clustergram and microarray of CGN plated on 3D NANS substrate for 8DIV. D, D2: Clustergram and microarray of CGN plated on 3D SANS for 8DIV. E. Map of gene probes and array locations.



**A2 time 0**



**B2 2D PL**



**C2 NANS**



**D2 SANS**



**E**

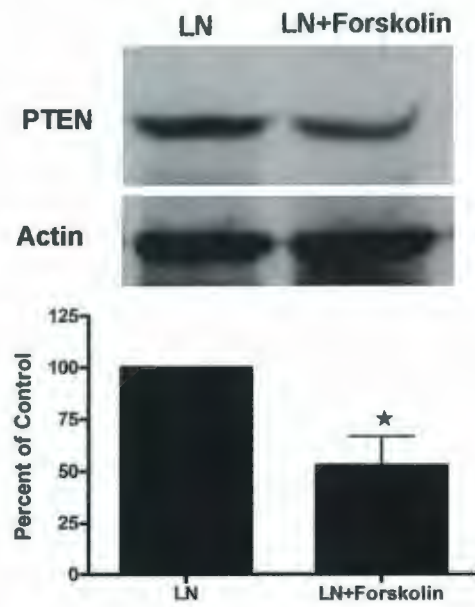
Ppia	Adams1	Adams5	Adams8_predicted	Ctnn1	Ctnna2_predicted	Cd44	Cdh1
1	2	3	4	5	6	7	8
Cdh2	Cdh3	Cdh4	Cdh5_predicted	Ctnn1	Col1a1	Col1a2	Col1a1
9	10	11	12	13	14	15	16
Col1a1	Col1a1	Col27a1	Col2a1	Col3a1	Col4a1	Col4a2_predicted	Col4a3
17	18	19	20	21	22	23	24
Col5a1	Col5a3	Col6a1_predicted	Col6a2	Col7a1_predicted	Col8a1_predicted	Col9a1	Cspg2
25	26	27	28	29	30	31	32
Ctgf	Ctnnb1	Ctnnd1_predicted	ECM1	Emilin1_predicted	Entpd1	Fbhl1_predicted	Fn1
33	34	35	36	37	38	39	40
Hapln1	Icam1	Itga1	Itga10_predicted	Itga11_predicted	Itga2	Itga3_predicted	Itga4
41	42	43	44	45	46	47	48
Itga5	Itga6	Itga7	Itga8	Itga9	Itga10	Itga11	Itga12
49	50	51	52	53	54	55	56
Itga13_predicted	Itga14	Itga15	Itga16	Itga17	Itga18	Itga19	Itga20
57	58	59	60	61	62	63	64
Itga21_predicted	Lama1_predicted	Lama2_predicted	Lama3	Lama4_predicted	Lama5	Lama6_predicted	Lama7
65	66	67	68	69	70	71	72
Lama8	Lama9	Mmp10	Mmp11	Mmp12	Mmp13	Mmp14	Mmp15_predicted
73	74	75	76	77	78	79	80
Mmp16	Mmp17_predicted	Mmp19_predicted	Mmp20_predicted	Mmp21	Mmp22_predicted	Mmp23	Mmp24
81	82	83	84	85	86	87	88
Mmp3	Mmp7	Mmp8	Mmp9	Ncam1	Ncam2	Postn_predicted	Postn
89	90	91	92	93	94	95	96
Selle	Selle	Selp	Sgce	Spock1	Spp1	Sx1	Sx1
97	98	99	100	101	102	103	104
Tgfb1	Thbs1	Thbs2	Thbs3	Timp1	Timp2	Timp3	Tnc
105	106	107	108	109	110	111	112
Vcam1	Vtn	RUC1B	Blank	Blank	AS1R1	AS1R2	AS1
113	114	115	116	117	118	119	120
Rpl32	Lcha	Aldoa	Aldoa	Gapdh	Gapdh	BAS2C	BAS2C
121	122	123	124	125	126	127	128

**Figure 10.11**

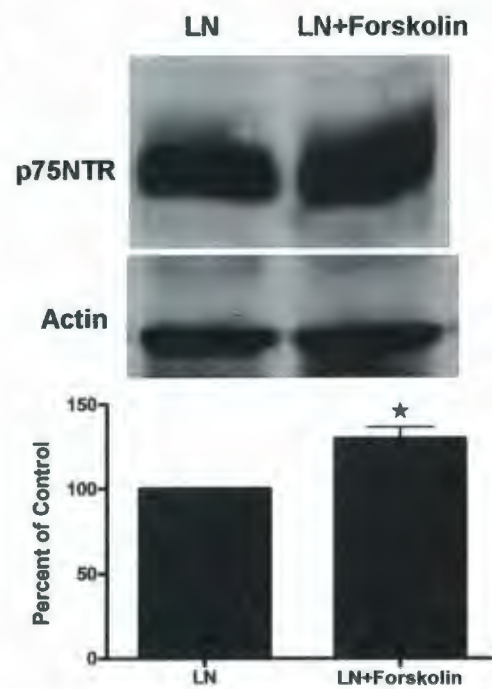
**Figure 10.12 Elevated cAMP decreases PTEN and increases p75NTR expression on LN.** PC12 cells were cultured on LN in the presence or absence of Forskolin (10 $\mu$ M) , an activator of cAMP. Addition of forskolin resulted in decreased PTEN (A) and increased p75NTR (B) expression. Values represent the mean expression of 3 independent experiments +/- SEM relative to actin. \* $p < 0.05$  (ttest).



**A**



**B**



**Figure 10.12**

4. There is strength in combining *in vitro* and *in vivo* techniques, and validation of the *in vitro* mechanisms presented here could be achieved by investigation of a variety of animal models. For example, the study of selective PTEN deletion in the mouse cerebellum could determine whether artificial regulation of p75NTR levels is sufficient to rescue architectural defects related to CGN migration, or if PTEN could have effects beyond the regulation of p75NTR that influence growth and migration of developing neurons.

## Chapter 11: Summary and Conclusions

Over the course of this series of studies, I have investigated signalling cascades initiated by neurotrophin and ECM stimuli independently and cooperatively in an attempt to characterize signalling associated with optimized axonal regeneration. My initial studies were all performed in PC12 cells, a sympathetic neuron model, and subsequent findings were confirmed in CNS neurons. The results obtained can be summarized as follows:

1. Molecular analysis of a series of PC12 derivative cell lines expressing either no TrkA (PC12nnr5) or TrkA mutated to abrogate Y490, Y785 or both Y490/785 phosphorylation sites of the TrkA cytoplasmic tail demonstrated a role for TrkA-associated phosphorylation sites in the regulation of p75NTR expression in both the presence and absence of NGF. This study demonstrated the critical role of the Y785 phosphorylation site to the NGF-induced upregulation of p75NTR, suggesting a role for Y785 interacting protein, PLC $\gamma$ .
2. A more detailed follow up study confirmed that the upregulation of p75NTR was due to the activation of TrkA by exogenously applied NGF, as selective activation of p75NTR itself had no impact on p75NTR expression, despite clear activation of the receptor as assessed by phosphorylation of JNK. I subsequently ruled out any involvement of the Ras-MAPK or PI3K signalling cascades using pharmacological

inhibition. As my initial study revealed an essential role for PLC $\gamma$  interaction site, Y785, in the regulation of p75NTR, I investigated the role of PLC $\gamma$  and associated downstream signalling in the induction of p75NTR expression in response to NGF stimulation. I used pharmacological inhibition of PLC $\gamma$  and both pharmacological inhibition and siRNAs against its downstream target PKC $\delta$  to confirm their involvement in the regulation of p75NTR expression in response to NGF stimulation. Finally, I investigated the nature of this signalling mechanism in CGN, which express TrkB and TrkC, but not TrkA, and was able to confirm that BDNF stimulation acts via TrkB-mediated PLC $\gamma$ -PKC $\delta$  signalling to induce p75NTR upregulation in CGN. These results provide a mechanism for the neurotrophin-induced upregulation of p75NTR, which may be of key importance to both developmental and regenerative axon growth.

3. A second factor well recognized to augment the growth response initiated by NGF-stimulation is a permissive substrate provided by the ECM. I therefore examined signalling initiated by neurotrophin and ECM ligands, both independently and cooperatively. As the phosphorylation of ERK is of paramount importance to a neurite outgrowth response in PC12 cells and sympathetic neurons, I used Western blotting to analyze the impact of individual and co-stimulation on ERK activation, and demonstrated that while LN and NGF both phosphorylate ERK, co-stimulation with both ligands results in enhanced phosphorylation than either stimulus alone. I further show that manipulation of ERK phosphorylation using wild-type or constitutively activated Ras constructs can induce neurite outgrowth, while dominant negative Ras is not associated with a growth response.



Because my initial studies illustrated that alterations in p75NTR expression were of potential importance to NGF-mediated signalling, I investigated the influence of LN on p75NTR expression. I demonstrate that a LN substrate downregulates p75NTR via both transcriptional and cleavage dependent processes, and that this downregulation is associated with a decrease in active Rho and enhanced regenerative growth. Conversely, overexpression of p75NTR, either wild-type or tagged, impaired neurite outgrowth on a LN substrate, and resulted in sustained Rho activation. The ability of LN to suppress p75NTR expression was demonstrated to be integrin dependent and furthermore was dependent upon LN-mediated upregulation of PTEN. PTEN expression displayed a consistently inverse relationship with p75NTR expression, and PTEN overexpression or siRNA manipulated p75NTR expression inversely. LN-mediated alterations in PTEN and p75NTR were further demonstrated to occur independently of the presence of TrkA and were correlated with the upregulation of Akt and GSK protein expression. Finally, I demonstrate the ability of LN to alter PTEN and p75NTR expression and promote enhanced regenerative growth in neonatal hippocampal neurons. These results characterize a novel ECM-mediated regulatory mechanism for both PTEN and p75NTR expression and provide evidence that LN promotes neurite outgrowth not only by enhancing the activation of signalling intermediates important for growth promotion, but also by suppression of a potentially inhibitory receptor, unliganded p75NTR (Figure 10.2).

4. Since ECM-integrin ligation and the subsequent manipulation of Rho activity could be critical to the promotion of cell migration in addition to neurite outgrowth, I

developed a novel assay to allow the study of the movement of a population of cells across an applied biological substrate with both qualitative and quantitative outcomes. By plating cells in a monolayer on one side of a small coverslip and subsequently inverting that coverslip over a LN-coated substrate, I was able to monitor and quantify the migration of cells across the LN substrate in the presence or absence of various stimuli or inhibitors, or after genetic modifications.

5. As my previous studies demonstrated that LN-induced alterations in PTEN and p75NTR expression could alter Rho activity for the promotion of neurite outgrowth in PC12 cells and neonatal hippocampal neurons, I investigated the presence of this cascade in CGN. While I discovered LN-induced changes in PTEN and p75NTR expression, and Rho activity levels were consistent with my previous findings, and indeed associated with early morphological changes, the signalling persisted beyond the period of neurite extension. A longer timecourse revealed that the differential expression of PTEN and p75NTR on LN versus PL was undetectable by 14 days *in vitro*, a timepoint correlating with the cessation of developmental migration. Motility analyses revealed enhanced migration on a LN substrate which correlated with increased PTEN and decreased p75NTR expression and again ceased by 14 days *in vitro*. PTEN siRNA and p75NTR overexpression inhibited CGN migration, while inhibition of Rho effector, Rock, both enhanced migration and rescued the migratory defects imposed by the aforementioned treatments. Based on the data collected, I concluded that LN-mediated PTEN upregulation and p75NTR downregulation promotes the migration of postnatal CGN and may thus be important to the development of proper cerebellar architecture.



6. I have demonstrated that PTEN played a role in the regulation of p75NTR expression. I subsequently investigated the mechanism by which this regulation may occur. Using a series of PTEN constructs mutated to eliminate all phosphatase activity (C124S) or selectively eliminate lipid phosphatase activity while retaining protein phosphatase activity (G129E), I determined that the ability of PTEN to downregulate p75NTR expression is dependent upon its protein phosphatase activity. Immunocytochemical analyses revealed a nuclear localization for PTEN on LN, and subsequent EMSA and ChIP analyses of transcription factor Sp1 activity revealed decreased DNA binding to the p75NTR promoter associated with both the LN substrate and the overexpression of PTEN. This mechanism provides an explanation for the decreased transcription of p75NTR. These studies were replicated in CGN with identical outcome. These results represent a detailed mechanism for the direct PTEN-mediated regulation of p75NTR expression.

7. My previous studies demonstrated that both Trk and PTEN play a role in the regulation of p75NTR expression. I therefore investigated if there was any interaction between Trk and PTEN. LN-induced PTEN upregulation had no impact on Trk expression. Conversely, TrkA reconstitution in Trk-deficient PC12nnr5 cells decreased PTEN expression in both the presence and absence of NGF. Mutation of the individual phosphorylation sites on the cytoplasmic tail of TrkA also influenced PTEN expression: loss of TrkA or Y785 site resulted in higher PTEN expression, while abrogation of Y490 phosphorylation site resulted in undetectable PTEN protein expression, and 10 fold lower

mRNA as determined by quantitative PCR. Trk-induced alterations in PTEN impacted the ability of the cells to respond to the LN substrate. The Y490F cell line did not downregulate p75NTR or decrease the level of active Rho in response to LN substrate, and subsequently displayed impaired growth and motility responses. Conversely Y785F cells displayed high PTEN expression and exhibited enhanced growth and motility in response to LN, relative to the parental cell line. These results appear to indicate a modulatory role for TrkA in the control of PTEN expression, with subsequent downstream influence over growth and migration responses, and lead us to conclude that the presence of PTEN protein is critical to the mediation of LN-induced modulation of the cytoskeleton in PC12 cells.

8. Since I previously demonstrated a role for ECM-integrin ligation in the regulation of PTEN expression, I investigated the kinetics and the mechanism underlying the LN-mediated induction of PTEN expression. Timecourse and concentration curve assessments indicated that PTEN mRNA and protein are induced within an hour exposure to LN substrate and that 1 $\mu$ g/ml LN is sufficient to elicit robust PTEN expression in both PC12 cells and CGN. I demonstrate that LN-mediated regulation of PTEN occurs in hepatoma-derived cell lines and is thus not a neuron specific response. We also expand our repertoire of substrates to demonstrate that integrin activation following exposure to LN, MG, FN or collagen results in PTEN induction, but exposure to CSPGs does not. Analysis of signalling intermediates necessary for PTEN induction in response to LN revealed roles for Src, FAK and ILK. Finally, LN-induced upregulation of transcription factor Egr-1 appears to play a critical role in this mechanism, as Egr-1 siRNA is sufficient



to prevent LN-induced PTEN upregulation. I conclude that the induction of PTEN is a robust response associated with integrin-mediated activation of Src, FAK, ILK and Egr-1, and that by virtue of downstream regulation of p75NTR and Rho activity, these steps may be critical components of the LN-induced promotion of axonal regeneration.

### **11.1 Conclusions**

To highlight my results, I will conclude by summarizing my findings:

1. The presence or absence of the individual phosphorylation sites of the cytoplasmic tail of TrkA modulates the constitutive expression of p75NTR.
2. Neurotrophin stimulation acts via TrkA to upregulate p75NTR expression. This occurs via the recruitment and phosphorylation of PLC $\gamma$  by phosphorylated Y785 on the Trk cytoplasmic tail, which results in the downstream activation of PKC $\delta$  and the subsequent upregulation of p75NTR.
3. ECM proteins signalling via integrin activation can autonomously activate signalling cascades essential for neurite outgrowth, and can cooperate with Trk signalling, resulting in enhanced activation of these cascades.
4. The ECM can act via integrin receptors to downregulate p75NTR expression by upregulating PTEN expression. This decrease in p75NTR results in a sustained

depression of active Rho, and as such, promotes regenerative growth of NGF-differentiated PC12 cells and neonatal hippocampal neurons.

5. The development of a novel motility assay allowed for the quantitation of migratory behaviour of cells plated on an applied biological substrate.

6. The ECM-mediated regulation of p75NTR via PTEN regulates the migratory abilities of postnatal CGN, and may direct their developmental migration.

7. LN induces nuclear localization of PTEN, where it acts to decrease the DNA binding ability of Sp1 to the p75NTR promoter via a mechanism dependent upon the protein phosphatase activities of PTEN. This decreased binding translates into decreased p75NTR transcription, and thereby downregulates p75NTR expression.

8. TrkA plays a role in the modulation of PTEN expression. TrkA expression inversely correlates with PTEN expression and the presence of particular phosphorylation sites on the cytoplasmic tail of Trk were able to modulate PTEN expression inversely to previously observed p75NTR expression levels.

9. Trk mutations that decreased the expression of PTEN impacted the ability of the cells to respond to LN by decreasing p75NTR and Rho activity, and ultimately determined the growth and migratory potential of the cells in response to a LN substrate.

10. LN-induced PTEN upregulation occurs rapidly following LN exposure and is dependent upon integrin signalling through Src, FAK and ILK, and ultimately depends upon the LN-induced alterations in transcription factor Egr-1.

**Overall conclusion:** From the results of these studies, I conclude that neuronal cells can respond to cues available from the extracellular environment, including neurotrophins and ECM molecules and alter p75NTR accordingly to promote an optimized growth response. In this way, the regenerative growth response following nerve injury is adaptable to context via the modulation of p75NTR expression, and the subsequent impact on the activity of Rho which has direct influence over the dynamics of the actin cytoskeleton.



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